THE UNIVERSITY of EDINBURGH

Title	Glucocorticoid modulation of macrophage phagocytosis of apoptotic neutrophils
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Qualification	PhD
Year	2002

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Glucocorticoid Modulation of Macrophage Phagocytosis of Apoptotic Neutrophils

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Presented for the degree of Doctor of Philosophy

The University of Edinburgh

November 2001

DECLARATION

This thesis was composed entirely by myself on the basis of work carried out under the supervision of Dr. Ian Dransfield and Dr. Adriano G. Rossi in the MRC Centre for Inflammation Research, University of Edinburgh

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Edinburgh, November 2001

ABSTRACT

Phagocytic clearance of apoptotic granulocytes is required for the successful resolution of inflammation, preventing the progression to a chronic state associated with development of fibrotic repair mechanisms and/or autoimmune responses. Macrophage capacity for phagocytosis of apoptotic cells can be modulated by a number of factors including cytokines and adhesion to extracellular matrix. We have hypothesised that alteration of macrophage phagocytosis could provide a strategy for increasing the rate of clearance in inflammatory disease. The aim of this thesis was to investigate the molecular mechanisms underlying control of macrophage phagocytosis of apoptotic cells. We have demonstrated that exposure of peripheral blood monocytes to the synthetic glucocorticoid dexamethasone "reprograms" monocyte/macrophage differentiation resulting in a macrophage phenotype with a marked augmentation in the phagocytosis of both apoptotic granulocytes and particles opsonized with low levels of IgG. Increase in phagocytic potential was not mediated by increased expression of putative "phagocytic receptors" proposed to be involved in apoptotic cell clearance. In addition, dexamethasone augmentation of apoptotic cell uptake could not be inhibited by blockade of receptor function with either soluble competitive ligands or monoclonal antibodies. Dexamethasone-treated macrophages were found to have altered morphology and actin organisation. In particular, loss of "podosome" structures was observed, possibly due to decreased recruitment of adhesion signalling molecules pyk2 and paxillin to focal contacts, and decreased expression of p130cas, a key adaptor molecule in integrin signalling. In addition, glucocorticoid treated cells showed increased activity of the Rho-family GTPase Rac, which has been previously shown to be important for phagocytosis and lamellipodia formation. Expression of p130cas and activation of the mitogen activated protein kinase, ERK are required for migration in a number of different cell types. Basal ERK activity was reduced in dexamethasone-treated monocyte/macrophages. We developed an *in vitro* "wounding" assay and found that despite the absence of basal ERK activity or p130cas expression in dexamethasone-treated macrophages, these cells were able to migrate.

To test the novel hypothesis that p130cas plays a pivotal role in regulation of macrophage phagocytic capacity, we generated a p130cas-HIV-TAT fusion protein. Full length p130cas sequence was subcloned and inserted into a TAT containing vector and then expressed in *E.coli*. Following purification of the fusion protein, preliminary studies were undertaken to investigate the effects of the protein on macrophage behaviour.

The morphological and functional changes induced by dexamethasone exposure were found to be influenced by the cytokine environment during monocyte-macrophage differentiation. Treatment of monocytes with the Th_1 cytokine IFNγ or the Th_2 cytokine IL-4 induced a multinucleated giant cell and dendritic cell like phenotype respectively. These macrophage phenotypes were characterised by increased podosome formation and decreased phagocytic capacity for apoptotic granulocytes. Whereas dexamethasone was found to prevent morphological changes induced by cytokine exposure, the increase in phagocytic capacity induced by dexamethasone was inhibited by differentiation in the presence of IFNγ and IL-4. Additionally, phenotypic analysis of cell surface markers differentially regulated by IFNγ and IL-4: CD64, CD14, ICAM-1 and the mannose receptor, revealed the generation of a distinct subset of monocyte/macrophages in the presence of dexamethasone plus cytokine as opposed to cytokine or dexamethasone alone. These studies have provided novel insight into the control of macrophage phagocytosis and will form the foundation for further studies investigating underlying molecular mechanisms.

ACKNOWLEDGMENTS

I would firstly like to thank all those people who gave blood, took blood or supplied me with cells, none of this work could have been undertaken without your generosity.

Secondly I would like to extend an enormous and sincere thank you to my supervisors: Dr. Ian Dransfield for his continued guidance, inspiration and advice throughout the last three years, and to Dr. Adriano Rossi for his encouragement, support and cakes. I would also like to thank Dr. Adam Lacey-Hulbert for his help and time regarding all things molecular biology.

During my four years in Edinburgh I've had some great times and learnt a lot about myself. None of this would have happened if it were not for some very special people who I would like to thank. Firstly those outside of work, in Edinburgh and further afield, my family and friends who supported me, no matter what I threw at them, especially Aziz for always looking out for me, and my flatmate Liz for her constant supply of wine. Secondly, I would like to send a big thankyou to all the people that make up the old Rayne Lab, and as it is now the CIR, most importantly Yattish, Julie, and Murray you are what make this such a fantastic and sociable place to work. Finally I would like to thank my fellow "usual suspects" Lorna, Annemieke, Carol and Jo, who over the past years have seen me through the laughs, the tears, and the beers - there's been plenty of them.

FUNDING

This work was supported by the Wellcome Trust and undertaken as part of their 4-Year PhD programme "The molecular and cellular basis of disease"

Laboratory facilities were provided by the MRC

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ABBREVIATIONS

βgal	β-galactosidase
ABC1	ATP-Binding cassette transporter
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C1q	Complement fragment 1q
C5a	Complement fragment C5a
CAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation (as in CD16)
CED	Ced death abnormal
CR	Complement receptor
DX	Dexamethasone
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
Fc	Fragment crystallizable
FcR	FC-receptor
FL-1	Log fluorescence-1
FMLP	*N*-formyl-methionyl-leucyl-phenylalanine
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GM-csf	Granulocyte-colony-stimulating factor
GRE	Glucocorticoid response element
GST	Glutathione-S-transferase
GTP	Guanine triphosphate
HA	hemagglutinin
HBSS	Hank's buffered saline solution
iC3bi	Complement fragment 3bi
ICAM-1	Intercellular adhesion molecule-1

IFNγ	Interferon-γ
IgG	Immunoglobulin
IL	Interleukin
IPTG	Isopropylthiogalactoside
ITAM	Immunoreceptor tyrosine based activation motif
LDL	Low density lipoprotein
LFA	Leukocyte function associated antigen
LPS	Lipopolysaccharide
M-1	Macrophage T_H1 type
M-2	Macrophage T_H2 type
MAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MHC	Major Histocompatability complex
MLCK	Myosin light chain kinase
MNC	mononuclear cell
MNGC	Mononuclear giant cell
Ni-NTA	Nickel-nitrilotriacetic acid
NO	Nitric oxide
PAGE	Polyacryamide gel electrophoresis
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PI 3-kinase	Phosphatidylinositol 3 kinase
PI	Propidium Iodide
PKC	protein kinase C
PLS	phopspho-L-serine
PS	Phosphatidylserine
PTB	Phosphotyrosine binding motif
RGDS	Arg-Gly-Asp-Ser peptide
RIPA	Radioimmunoprecipitation assay
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulphate

SH-2	Src homology-2 domain
SR-A	Scavenger receptor class A
SREC	Scavenger receptor on Endothelial cells
TBS	Tris buffered saline
TGFβ	Transforming growth factor beta
T_H1	Inflammatory CD4 T cells
T_H2	Helper CD4 T cells
TNFα	Tumour necrosis factor
WASp	Wiskott-Aldrich Sydrome protein

CHAPTER 1: INTRODUCTION

Inflammation: An overview

The inflammatory response has evolved as a defence mechanism against microbial infection or tissue injury. Immune cell recognition of "foreign" material induces the release of chemokines and other inflammatory mediators that increase local blood flow and vessel permeability, and promote the recruitment of immune cells to the site of injury. Accumulation of fluid and inflammatory cells within tissues produces the characteristic *calor, dolor, rubor,* and *tumor* (heat, pain, redness and swelling) of an inflammatory response (Gallin *et al.*, 1992).

Neutrophils are the most abundant white blood cell in the circulation and the earliest to be recruited to inflammatory sites in response to chemoattractants such as IL-8 released by monocytes, macrophages and fibroblasts (Follin et al., 1991; Kunkel *et al.*, 1991; Smith *et al.*, 1991) (figure 1). Their primary function is phagocytosis and destruction of "foreign" material, by the release of pre-formed enzymes and proteins from intracellular granules into intracellular compartments containing phagocytosed material, or extracellularly onto the pathogen, such as cationic proteins, lysozme and acid hydrolases. Additionally, neutrophils are able to generate highly microbicidal reactive oxygen species (Gudmundsson and Agerberth, 1999). Neutrophils also have the capacity to synthesise a number of cytokines which have systemic effects, increasing temperature (Netea *et al.*, 2000) and inducing the further recruitment of inflammatory cells (Doherty *et al.*, 1988). The importance of neutrophil function in mediating acute inflammation is emphasised by the recurrent bacterial infections suffered by patients with deficiencies in either neutrophil number: neutropaenia (Bodey *et al.*, 1966), or neutrophil function: leukocyte adhesion deficiency (Anderson and Springer, 1987), Chediak-Higashi syndrome (Rausch *et al.*, 1978) and chronic granulomatous disease (Thrasher *et al.*, 1994).

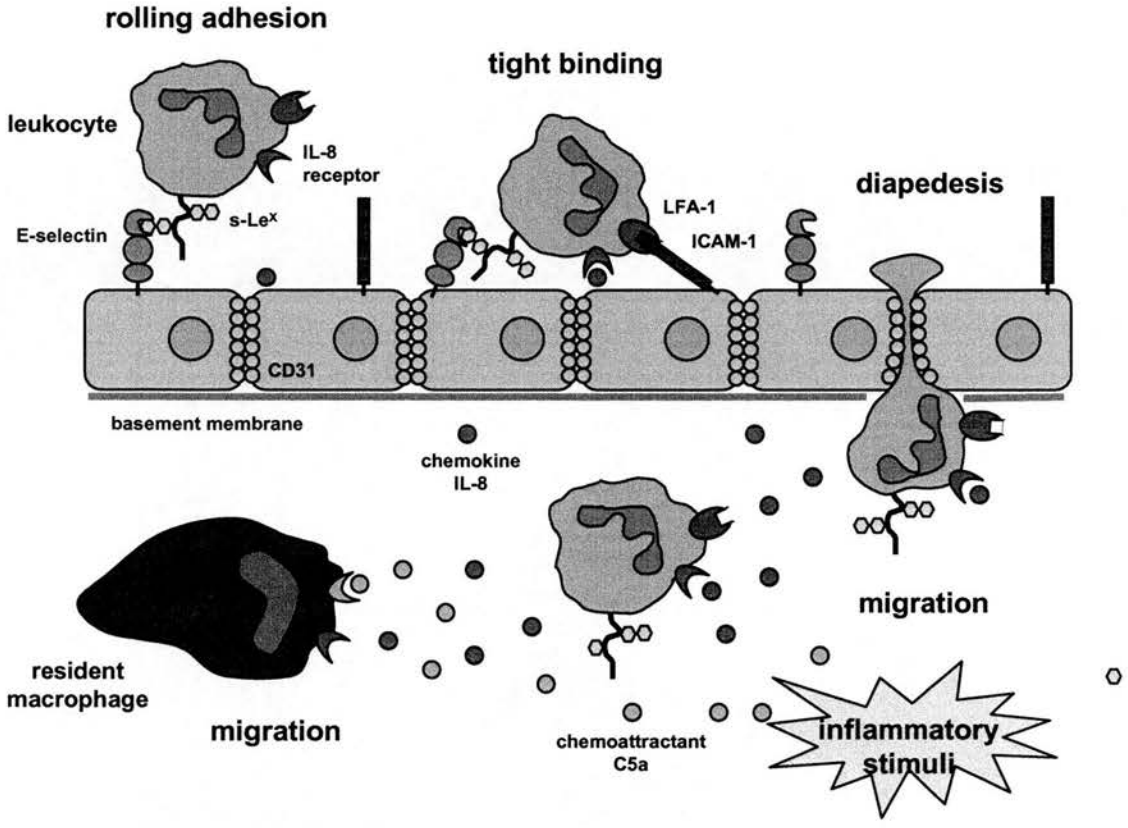


Figure 1. Recruitment of inflammatory cells. Chemokines induce extravasation of leukocytes from the blood stream into tissues. Sialyl-Lewis^x moieties on neutrophils bind to P-selectin and E-selectin on the surface of endothelial cells, externalised in response to inflammatory mediators such as leukotriene B_4, C5a, histamine, LPS and TNFα. This mediates weak adhesion and rolling of the granulocyte along the surface of the endothelial wall. Rolling is inhibited by the induction of strong adhesion from the interaction of LFA-1 and complement receptor 3 (CR3) on the leukocyte to ICAM-1 on the surface of endothelial cells which is also externalised in response to TNFα. Normally the LFA-1 and CR3 interactions are low affinity, however IL-8 induces changes in the conformation and distribution of these integrins promoting binding. Leukocytes then undergo extravasation through the endothelial wall via further LFA-1 and CR3 interactions, plus homotypic binding of PECAM (CD31) present on both leukocytes and the intracellular junctions of endothelial cells. Finally the cell penetrates the basement membrane by the release of proteolytic enzymes in a process known as diapedesis. Monocyte migration follows a similar pattern except VLA-4 and VCAM-1 appear to be important for binding. Tissue macrophages also migrate to the inflammatory stimulus in response to chemokines, and are often initiators of the inflammatory cascade after engulfment of particulate matter.

Macrophages represent the sentinels of the immune system with the capacity to ingest and destroy infectious agents, by the release of enzymes and toxic metabolites, extracellularly and into intracellular compartments. These include nitric oxide, superoxide radicals, lysozyme and acid hydrolases (MacMicking *et al.*, 1997; Nathan, 1987; Nathan and Hibbs, 1991). Macrophages initiate T-cell responses by antigen presentation, and act as effector cells for both humoral and cell-mediated immune responses (Uanue and Allen, 1987). They arise from blood monocytes recruited into tissues in response to a wide variety of chemotactic agents for example MCP-1 (Monocyte Chemotactic Protein), RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and C5a, a component of the complement cascade. Macrophages are located throughout the different organs and tissues of the body and the connective tissue around the basement membrane of small blood vessels. They are particularly concentrated in the lung (alveolar macrophages), liver (Kupffer cells) and the lining of spleen sinusoids and lymph node medullary sinuses, where they are strategically placed to "filter" foreign antigen. Other cells of the monocyte/macrophage lineage include mesangial cells in the kidney glomerulus, brain microglia and osteoclasts in bone. The early inflammatory response enables either complete clearance of the pathogen or containment of the infection before the induction of adaptive immunity. Cytokines produced during this period help determine the subsequent development of the adaptive immune response and can determine if this is predominantly T cell mediated or humoral (Mills *et al.*, 2000; Mosmann and Sad, 1996).

Whilst the inflammatory response has the capacity to clear a large proportion of infections, the cellular functions of neutrophils and macrophages have the potential to inflict "bystander" damage on neighbouring cells, thereby exacerbating inflammation and inducing tissue damage and scarring, characteristic of chronic inflammatory conditions such as asthma, emphysema and rheumatoid arthritis (Gadek, 1992; Haslett *et al.*, 1994; Robinson *et al.*, 1992; Weiss, 1989; Woolley *et al.*, 1996). Inflammatory responses must therefore be highly regulated, at both the level of the recruitment of cells to the tissue and also their activation state. Priming of granulocytes with inflammatory mediators such as LPS induces a heightened

parasiticidal/microbicidal activity in response to fMLP (Guthrie *et al.*, 1984). Similarly activation of macrophages by IFNγ prior to TNFα exposure increases nitric oxide production (Erwig *et al.*, 1998; Lake *et al.*, 1994). Although it is often assumed that chronic inflammation occurs as a result of uncontrolled pro-inflammatory events, there is much evidence to suggest that failure or inefficient resolution processes are responsible for tipping the balance toward persistent inflammation (Akbar and Salmon, 1997; Haslett *et al.*, 1994).

Resolution of inflammation

In order for resolution of inflammation to occur with the return to normal tissue homeostasis, all the processes involved in the initiation of inflammation must be reversed. This includes removal of stimuli responsible for initiating and maintaining the inflammatory response such as bacterial and cellular debris and pro-inflammatory cytokines, a cessation of granulocyte and monocyte migration into the tissue, plus an active clearance of extravasated cells. Deletion of granulocytes is thought to be mediated by the induction of a specific form of cell death, apoptosis, followed by phagocytosis of the cell corpse by macrophages (Haslett, 1992; Newman, 1982; Savill *et al.*, 1989b), (figure 2). Dysregulation in the induction of granulocyte apoptosis has been implicated in the pathogenesis of adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, ulcerative colitis, rheumatoid arthritis, asthma, and other allergic diseases (Haslett *et al.*, 1994; Meagher *et al.*, 1992; Weiss, 1989; Woolley *et al.*, 1996).

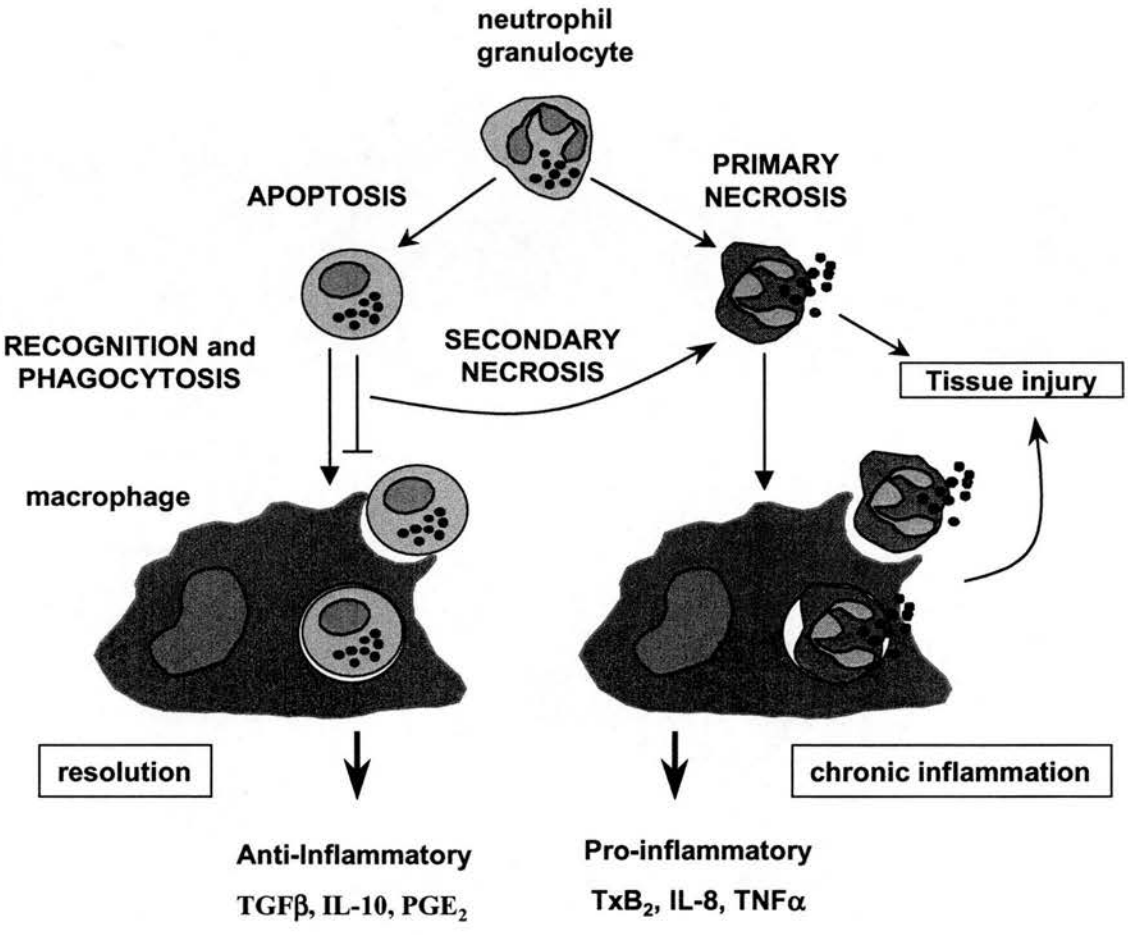


Figure 2. Clearance of inflammatory cells.
Resolution of inflammation requires the "safe" removal of extravasated cells. This is mediated by induction of granulocyte apoptosis, followed by phagocytosis by macrophages. During apoptotic cell death, membrane integrity is maintained, preventing the release of granule contents onto surrounding tissues. Phagocytosis of apoptotic cells, unlike uptake of other particles, is non-phlogistic, and actually induces the release of anti-inflammatory mediators that further promote resolution. In contrast, necrotic cell death results in the release of the formidable array of granulocyte cytotoxic contents, inducing tissue damage and further recruitment of inflammatory cells. Furthermore, uptake of necrotic debris potentiates the inflammatory response inducing release of pro-inflammatory cytokines by the macrophage. Residual apoptotic cells not cleared by phagocytes undergo secondary necrosis, resulting in a similar chronic inflammatory state.

Apoptosis

Apoptosis is an evolutionarily conserved mechanism executed by a family of proteases called caspases that cleave targets at specific aspartic acid motifs (for review see Wolf and Green, 1999). Caspases function either as upstream initiators of the apoptotic program or as its downstream effectors. In non-apoptotic cells caspases are present in an inactive pro-form which requires cleavage by an active caspase for activity, the active protease can then cleave down stream caspases creating a proteolytic cascade which functions to amplify the initial apoptotic stimulus. Initial caspase cleavage is thought to occur via " proximity mechanisms" (Salvesen and Dixit, 1998). Ligation of cell surface "death" receptors induces receptor clustering and recruitment of intracellular proteins and caspases into a "death inducing complex". In their inactive form, caspases have a low basal activity, which is not normally sufficient for induction of the proteolytic cascade, however within the proximity of the death-inducing complex, full caspase activation occurs. The second mechanism thought to mediate caspase activation involves release of cytochrome c from the mitochondrion, regulated by members of the Bcl-2 family of proteins (Kroemer and Reed, 2000). Cytosolic cytochrome c binds and activates Apaf1, forming the apoptosome and subsequent caspase activation. The caspase cascade then executes the apoptotic program resulting in cleavage of a number of cytosolic proteins such as the structural proteins laminin, keratin, p130cas, actin, FAK, and PARP (Kook et al., 2000). In addition caspases are able to further activate or inactivate pro and anti-apoptotic proteins such as Bcl-2, other pro-caspases, and ICAD, responsible for induction of DNA fragmentation. A number of apoptosis regulators exist (for review see Reed, 2001), these include protein kinases such as Akt and IκB kinase which both function to maintain transcription of anti-apoptotic genes, the IAP family of proteins, which inhibit caspase activation, and the Bcl-2 family of proteins. The Bcl-2 family consist of both pro and anti apoptotic molecules which form homodimers; the relative amounts of these molecules determines the resistance of cells to apoptotic stimuli such as growth factor deprivation, hypoxia, radiation, oxidants, and elevated $[Ca]^{2+}$. Thus caspase cleavage of cytosolic proteins during apoptosis terminates many cellular activities, inducing the down regulation of

granulocyte cell function observed during apoptosis and the resolution of inflammation, but maintains membrane integrity (Savill *et al.*, 1989b; Whyte *et al.*, 1993). Stimulated granule release and phagocytosis are inhibited due to an inhibition of membrane trafficking, and specific changes in the expression of cell surface molecules occur which prevent further stimulation of the granulocyte by inflammatory mediators. In the neutrophil, fMLP receptors are partially lost and there is an almost complete depletion of the cell surface IgG receptor FcRγIII (CD16), in addition to L-selectin, TNF receptors p55 and p75, and CD43 (Dransfield *et al.*, 1994; Dransfield *et al.*, 1995; Meagher *et al.*, 1992). Receptor shedding occurs during granulocyte activation suggesting that pathways engaged during activation (of the neutrophil) also function during its death throes. Induction of a cell that is functionally "isolated" may be important to limit local tissue injury before apoptotic cell clearance by macrophages. A number of stimuli have been characterised as regulators of granulocyte viability (table 1) (for review see Ward *et al.*, 1999). These include inflammatory agents such as LPS and GM-CSF, which inhibit neutrophil apoptosis (Colotta *et al.*, 1992; Cox *et al.*, 1992; Haslett *et al.*, 1991; Lee *et al.*, 1993; Yamamoto *et al.*, 1993), TNFα, nitric oxide donors, and ligation of Fas which promote granulocyte cell death (Matsumoto *et al.*, 1995; Murray *et al.*, 1997; Ward *et al.*, 2000). Interestingly work by Brown and colleagues have described how exposure of macrophages to non-particulate stimuli can induce the induction of apoptosis in bystander leukocytes via the Fas death pathway (Brown and Savill, 1999), initiating the "safe" clearance of infiltrated neutrophils.

Granulocyte	Apoptosis delaying agents
Neutrophil	Agents that increase cytosolic cAMP
	Agents that increase $[Ca^{2+}]_i$
	Glucocorticoids
	Neutrophils agonists (LPS, FMLP, PAF, LTB_4, C5a, GM-CSF, IL-6, IL-8)
	Hypoxia
Eosinophil	Agents that increase cytosolic cAMP
	Eosinophil agonists (IL-5, GM-CSF)
	Apoptosis accelerating agents
Neutrophil	NO donors
	Activating Fas antibodies, FasL
	TNFα
	Protein synthesis inhibitors
	NF-κB inhibitors
Eosinophil	Activating Fas antibodies, FasL
	Agents that increase $[Ca^{2+}]_i$
	Glucocorticoids
	NF-κB inhibitors

Table 1. Regulation of granulocyte survival.
Abbreviations: $[Ca^{2+}]i$, intracellular free calcium concentration; C5a, complement fragment C5a; Fas, Fas ligand; FMLP, formyl-methionylleucylphenylalanine; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; LTB_4, leukotriene B_4, NF-κB, nuclear factor κB; NO, nitric oxide; PAF, platelet-activating factor; TNFα, tumour necrosis factor α. Adapted from Ward et al., 1999.

Phagocytosis

Phagocytosis of the "inert" apoptotic particle can itself promote resolution. Unlike ingestion of IgG opsonized particles which promote the release of pro-inflammatory cytokines, uptake of apoptotic cells is non-phlogistic, and actually induces the release of anti-inflammatory mediators such as TGFβ and PGE_2, and inhibits subsequent TNFα release in response to inflammatory stimuli (Fadok *et al.*, 1998a; Voll *et al.*, 1998). This "re-setting" of macrophage activation can be mimicked by ligation of macrophage receptors thought to mediate uptake of apoptotic cells (table 2). For example CD36, its bridging molecule thrombospondin (Fadok *et al.*, 1998b), and the recently identified receptor for phosphatidylserine exposed on the surface of apoptotic cells (Fadok *et al.*, 2000). IFNγ and LPS are the major mediators of classical macrophage activation inducing production of pro-inflammatory cytokines, whereas IL-4, -10, and –13, TGFβ and glucocorticoids have been shown to "alternatively" activate macrophages inhibiting the production of pro-inflammatory cytokines, NO and reactive oxygen species (Goerdt and Orfanos, 1999; Mills *et al.*, 2000; Stein *et al.*, 1992). Alternatively-activated macrophages are thought to metabolise arginine to ornithine, as opposed to NO (Mills *et al.*, 2000; Munder *et al.*, 1998; Munder *et al.*, 1999). As a precursor of polyamines, ornithine promotes cell replication, facilitating cell renewal during wound healing (Williams-Ashman and Canellakis, 1979). Thus, changes in the cytokine environment mediated by clearance of apoptotic cells can therefore program infiltrating monocyte/macrophages and further influence the inflammatory outcome.

In contrast to the induction of apoptotic cell death and subsequent clearance by macrophages, necrotic cell death and clearance of necrotic cells evokes pro-inflammatory responses (Cocco and Ucker, 2001; Fadok *et al.*, 2001a; Stern *et al.*, 1996). Cell membrane integrity is not maintained, resulting in the leakage of the cell's toxic contents, and in addition phagocytosis induces macrophage production of inflammatory mediators. Inflammatory diseases are therefore likely to arise from failure or inefficiency of normal resolution mechanisms. There are a number of steps in the clearance pathway where dysregulation could promote chronic inflammation,

by altering the balance between apoptotic and necrotic cell death. Necrotic cells are present even in resolving inflammation. Whether these arise from failure to induce apoptosis or due to the induction of secondary necrosis in non-cleared apoptotic cells is not known, or how many necrotic cells are required to be present to switch the balance from resolving to non-resolving inflammation. This is illustrated by *in vivo* triggering of Fas which induces massive apoptotic cell death in the liver of mice, overwhelming macrophage clearance mechanisms and resulting in secondary necrosis, tissue damage and ultimately organ failure (Ogasawara *et al.*, 1993). Similarly, induction of bronchial and epithelial cell apoptosis in the rat lung by inhalation of Fas mAb results in a necrotic lesion, inflammation and scarring (Hagimoto *et al.*, 1997). The efficiency of clearance of apoptotic cells is likely to influence the number of necrotic cells present in tissues in addition to modulation of the death program itself. Much work has been undertaken to understand the mechanisms modulating inflammatory cell survival, however, unless increased apoptosis is paralleled by an augmentation of macrophage clearance it is possible that beneficial effects may be reduced. Recent work has described how the act of engulfment can trigger the final death throes of a cell (Hoeppner *et al.*, 2001; Reddien *et al.*, 2001). Thus, engagement of the full apoptotic program would be delayed until phagocyte engulfment begins, reducing the possibility of secondary necrosis. Modulation of macrophage phagocytic capacity therefore represents a major therapeutic target for the treatment of inflammatory disease.

Macrophage clearance of apoptotic cells

Clearance of apoptotic cells by macrophages is regulated at a number of levels. Firstly, the exposure of "eat-me" signals on the apoptotic cell surface and interaction of specialised receptors expressed by macrophages and other phagocytic cells function to identify and "tether" the particle prior to engulfment. "Tethering" may be enhanced by substances that opsonize apoptotic cells, providing additional cell-cell interactions. Secondly, engagement of the apoptotic particle evokes intracellular

signalling pathways that induce cytoskeletal changes mediating the internalisation of the apoptotic cell and its subsequent processing.

Apoptotic cell ligands

The absence of internalised viable cells within phagocytic populations suggests that uptake of apoptotic cells is mediated in response to an "eat me" signal induced by the progressing apoptotic program. However, the observation that macrophages internalise inert particles could argue that phagocytosis of apoptotic cells proceeds in response to the loss of a viable cell markers. Earlier I have described the decrease in expression of certain neutrophil cell surface molecules during apoptosis (Dransfield *et al.*, 1994; Dransfield *et al.*, 1995) implying that mechanisms for maintenance of receptor numbers are compromised or proteolytic shedding is employed. Such protease activity would be required to be specific for apoptotic or necrotic cell death. Proteolytic shedding of surface receptors during apoptosis has been observed in myeloid cell lines (Brown *et al.*, 1996), but whether this represents a fundamental feature of apoptosis in different cell types remains to be determined. One possibility is that the presence of proteolytic "stubs" of shed receptors could contribute to a "signal" for phagocytosis. Differences in the apoptotic cell ligand may determine the cellular response to ingestion, for example the release of pro-inflammatory cytokines after necrotic cell or latex bead uptake, or release of anti-inflammatory mediators as described during apoptotic cell phagocytosis (Fadok *et al.*, 1998a; Voll *et al.*, 1998). Ligation of apoptotic cell receptors on phagocytes during clearance of necrotic cells could "fool" the macrophage into eliciting anti-inflammatory responses promoting resolution of the necrotic lesion.

A number of potential apoptotic cell markers have been identified, most of which depend on specific modification of cell surface molecules by proteolysis, oxidation, or changes in glycosylation. Non-enzymatic glycosylation of surface proteins to form advanced glycosylation end products (AGE) induces binding of specific receptors termed RAGE (Schmidt *et al.*, 1994). RAGE is also able to bind β-amyloid fibrils associated with the neuropathology of Alzheimer's disease (Schmidt

et al., 2000). Failure to clear these modified proteins may provoke inflammatory responses observed in neurological tissue from Alzheimer's sufferers. However, inhibition of macrophage phagocytosis with specific polyanionic ligands does not support a potential role for AGE in the clearance of apoptotic cells (Savill *et al.*, 1989a), additionally the kinetics of appearance of AGE on senescent cells is likely to be too slow for recognition of apoptotic cells by phagocytes.

Other modifications of cell surface molecules thought to induce phagocytosis include desialylation of lectins (Duvall *et al.*, 1985; Pradhan *et al.*, 1997) and the alteration of the adhesion molecule ICAM-3 (Gregory *et al.*, 1998; Moffatt *et al.*, 1999). Early studies indicated that cellular microelectrophoretic mobility was reduced on apoptotic mouse thymocytes, suggestive of changes in the net negative charge of cell membranes from changes in surface proteins (Morris *et al.*, 1984). This corresponded to reduced binding of lectins and mAb observed in flow cytometric analysis (Morris *et al.*, 1984). High levels of mannose, N-acetylgalactosamine, and galactose can be detected on apoptotic human peripheral blood lymphocytes using fluoresceinated lectins as probes and have been implicated in clearance of apoptotic cells (Dini *et al.*, 1992). Flow cytometric analysis of binding of a panel of conjugated lectins to apoptotic neutrophils suggests that exposure of these carbohydrate moieties may not be a general feature of apoptosis on all cell types (Hart *et al.*, 2000). Screening of the effects on apoptotic cell uptake of a large panel of mAb revealed that blockade of certain epitopes of ICAM-3 (CD50) on apoptotic B cells prevented uptake. One possibility is that ICAM-3 is modified following induction of apoptosis in a manner that confers binding to CD14, a cell surface protein previously implicated in the uptake of apoptotic cells (Devitt *et al.*, 1998; Moffatt *et al.*, 1999).

The most extensively characterised apoptotic cell marker is the externalisation of amino phospholipids including phosphatidylserine (PS) (Martin *et al.*, 1995) and phosphatidyl ethanolamine (Emoto *et al.*, 1997). In viable cells, PS is located on the cytoplasmic face of the cell membrane (Op den Kamp, 1979; Schroit and Zwaal, 1991; Zwaal and Schroit, 1997). During induction of the apoptotic program "flip

flop" of PS occurs exposing it on the surface of the cell (Bratton *et al.*, 1997; Martin *et al.*, 1996). This is mediated by specific scramblases, intracellular proteases such as calpains and caspases, and transglutaminases (Bratton *et al.*, 1999, and references therein), plus the ABC1 transporter (see below) (Hamon *et al.*, 2000). Pharmacological inhibition of scramblase function prevents uptake of apoptotic cells (Fadok *et al.*, 2001b) suggesting that although other apoptotic ligands may promote phagocytosis by either tethering or acting in a co-stimulatory fashion, PS exposure is obligatory. Identification of the only phagocytic receptor in *C. elegans* as a possible receptor for modified lipid (Zhou *et al.*, 2001) suggests that membrane asymmetry represents a conserved signal for cell death. Interestingly, PS exposure has been described to be a specific marker for apoptotic cell death and has been proposed to be a molecular "switch" for the release of anti-inflammatory cytokines associated with their engulfment (Henson *et al.*, 2001). However, a number of reports have demonstrated its involvement during clearance of necrotic particles (Cocco and Ucker, 2001) and additionally during caspase-independent cell death (Brown *et al.*, 2000; Hirt *et al.*, 2000). CD36, an alternative PS receptor, has been shown to bind PS in addition to other oxidised lipids associated with apoptosis (Fadok *et al.*, 1998b; Tait and Smith, 1999). Co-operation between the PS receptor and additional stimulatory molecules may be required for the induction of the specific responses described by Fadok and colleagues (Fadok *et al.*, 1998b).

Surface molecule	Phagocyte	Apoptotic particle
$\alpha_v\beta_3$/CD36/thrombospondin		
αvβ3 / thrombospondin/ CD36	Human monocyte-derived macrophage (Savill et al., 1990; Savill et al., 1992)	Neutrophil
αvβ3/ thrombospondin	Human fibroblast (Hall et al., 1994)	Neutrophil
	Mouse bone marrow-derived macrophage (Ren et al., 2001)	Neutrophil
αvβ3/ thrombospondin/ proteoglycans	Human glomerular mesangial cell (Hughes et al., 1997)	Neutrophil
αvβ3	Mouse bone marrow-derived macrophage (Fadok et al., 1992a)	Lymphocyte
	J774 mouse macrophage (Pradhan et al., 1997)	
	Human dendritic cell (Rubartelli et al., 1997)	Jurkat
CD36	Human THP-1 macrophage	Lipid microspheres
	House J774 macrophage (Tait and Smith, 1999)	
	Microglia (Witting et al., 2000)	Neuronal cell
	COS-7 cell (ectopic expression) (Ren et al., 1995)	Neutrophil, Lymphocyte, Fibroblast
CD36/αvβ3	Small airway epithelial cell (Walsh et al., 1999)	Eosinophil
CD36/αvβ5	Human dendritic cell (Albert et al., 1998)	Monocyte
αvβ5	Rat retinol pigment epithelium cell (Finnemann and Rodriguez-Boulan, 1999)	Photoreceptor outer segment particles
Phosphatidylserine receptors		
"putative" PS receptor	Mouse peritoneal macrophage (Fadok et al., 1992b)	Lymphocyte
	Stimulated bone marrow-derived macrophage (Fadok et al., 1992a)	
	Human PMA-stimulated THP-1 cell (Fadok et al., 1992b)	Lymphocyte
	J774 mouse macrophage (Pradhan et al., 1997)	Lymphocyte
	Rat vascular smooth muscle cell (Bennett et al., 1995)	Vascular smooth muscle cell
	Rat sertoli cell (Shiratsuchi et al., 1997)	Spermatogenic cell
	Kupffer cells (Shi et al., 2001)	Neutrophil
	Microglia (Witting et al., 2000)	Neuronal cells
Human PSR	Stimulated human monocyte derived macrophages (Fadok et al., 2000)	Jurkat
	Fibroblasts (Fadok et al., 2001)	Jurkat, HL-60
Scavenger Receptors		
SR-A	Mouse peritoneal macrophage	Thymocyte
	Thymic macrophage (Platt et al., 1996)	
	Human monocyte-derived macrophage (Brown et al., 2000)	Platelet
SR-B1	Rat sertoli cell (Shiratsuchi et al., 1999)	Spermatogenic cell
	Rat thecal cell (Svensson et al., 1999)	Granulosa cell
OxLDL receptor	Mouse peritoneal macrophage (Chang et al., 1999)	Thymocyte
Lox-1	Endothelial cell (Oka et al., 1998)	HL-60 Jurkat
CD68	Mouse peritoneal macrophages (Sambrano and Steinberg, 1995)	Thymocytes
Pattern Recognition Receptors		
CD14	Human monocyte-derived macrophage (Devitt et al., 1998; Flora and Gregory, 1994; Moffatt et al., 1999)	Lymphocyte, Neutrophil
	J774 mouse macrophage (Pradhan et al., 1997)	Lymphocyte
	Peritoneal macrophage (Pradhan et al., 1997)	Lymphocyte
Mannose/fucose receptor	Human fibroblast (Hall et al., 1994)	Neutrophil
Lectin	Mouse peritoneal macrophage (Duvall et al., 1985; Pradhan et al., 1997)	Thymocyte, Lymphocyte
	Endothelial cell (Dini and Carla, 1998)	Liver cell
	Small airway endothelial cell (Walsh et al., 1999)	Eosinophil
Asioglycoprotein receptor	Rat liver cell (Dini et al., 1992)	Liver cell
	Liver endothelial cells (Dini et al., 1995)	
	Kupffer cell (Falasca et al., 1996)	
	Microglia (Witting et al., 2000)	Neuronal cells
Adhesion receptors		
β_1-integrin	Human monocyte	Endothelial cell
	U937 monocytes (Schwartz et al., 1999)	
CD44	Human monocyte-derived macrophage (Hart et al., 1997)	Neutrophil
Receptor tyrosine kinase		
Mer	Mouse bone marrow derived macrophage (Scott et al., 2001)	Mouse thymocyte
ATP-binding cassette transporter (ABC1)	Mouse peritoneal macrophage (Luciani and Chimini., 1996)	Thymocyte
Opsonins (Receptor)		
C3bi (CR3/CR4)	Human retinoic acid differentiated THP-1 cell (Takizawa et al., 1996)	Jurkat
	Human monocyte derived macrophage (Mevorach et al., 1998)	Thymocyte
C1q (C1qR)	Cells in renal glomeruli (Botto et al., 1998)	Cells in renal glomeruli
C1q/Mannose binding lectin (calreticulin/CD91)	Human monocyte derived macrophages (Ogden et al., 2001)	Jurkat
Surfactant protein A (CD91?)	Rat alveolar macrophage (Schagat et al., 2001)	Rat neutrophil,
β_2-glycoprotein (β_2-glycoprotein R)	Mouse peritoneal macrophage (Balasubramanian et al., 1997)	Jurkat
Gas6 (Mer)	Mouse peritoneal macrophages (Ishimoto et al., 2000)	Thymocyte

Table 2. Receptors mediating uptake of apoptotic cells

Opsonins

A growing number of soluble molecules have been shown to promote apoptotic cell uptake by opsonization of apoptotic particles (table 2). The complement components C1q and iC3b bind to the surface of apoptotic cells promoting uptake via C1q receptors or CR3/CR4 (Korb and Ahearn, 1997; Mevorach *et al.*, 1998; Takizawa *et al.*, 1996). Susceptibility to the autoimmune disease systemic lupus erythematosus has been linked to C1q deficiency. An excess of free non-ingested apoptotic cells are found in the inflamed glomeruli seen in mice deficient in C1q (Botto *et al.*, 1998). Self antigens which are targets for autoimmune disease have been shown to be present on the surface of late stage apoptotic cells, and apoptotic blebs (Gilligan *et al.*, 1996; Kalden, 1997). In addition immature dendritic cells have recently been shown to present peptides derived from apoptotic cells ingested via $\alpha_V\beta_5$ integrins in both MHC Class I and MHC Class II (Albert *et al.*, 1998a; Inaba *et al.*, 1998). However, in the absence of co-stimulatory molecule expression presentation of peptides is thought to induce a toleragenic response to self-antigens. Macrophages were shown not to be able to present material from apoptotic particles upon phagocytosis via CD36 and $\alpha_V\beta_3$. Failure of efficient clearance by C1q deficiency may result in uptake of particles at the late apoptotic or necrotic stage. This may represent a "danger" signal inducing differential processing and presentation of particle fragments resulting in autoimmunity. CD14 functions as a receptor for LPS as well as apoptotic cells (see below), uptake of bacterial products at the same time as apoptotic cells may also result in presentation of bacterial and self antigens via class II. As macrophages are proficient at stimulating T cell mediated immunity to pathogens, transient autoimmune responses may also occur which contribute to the tissue damage acquired during the course of certain inflammatory responses.

Receptors for recognition of apoptotic cells

Much information on the intracellular mechanisms required for the uptake of apoptotic cells have arisen from the study of phagocytosis in the nematode worm *C. elegans*. However, study of this model organism has not enabled the elucidation of

the extracellular mechanisms required for recognition and initiation of phagocytosis of apoptotic particles by mammalian cells. Whilst the genetic approach can identify molecules involved in global uptake mechanisms it does not address functional associations or distribution of phagocytic receptors, or the contribution of post-translational modifications such as phosphorylation and glycosylation on apoptotic cell uptake. A putative nematode phagocytic receptor has recently been identified as a transmembrane protein thought to recognise altered lipid products with homology to the mammalian protein SREC (Scavenger Receptor of Endothelial Cells) a member of the scavenger receptor family (Zhou *et al.*, 2001). Genetic screens have not suggested the presence of other receptors. In contrast to the worm at least seven distinct families of cell surface proteins have been implicated in the recognition of apoptotic cells in mammalian systems (table 2). The putative receptors range from integrin molecules previously described for their role in adhesion signalling, receptors which mediate recognition of pathogens in innate immunity, to proteins which mediate the uptake and processing of lipid products. Mammalian "recognition" receptors appear to have dual roles in both clearance of apoptotic cells and other cellular functions required to maintain tissue homeostasis. Although multiple receptors and subsequent redundancy complicate investigation into uptake mechanisms, the duality of their function enables study of these receptors outwith their phagocytic capacities to help understand their cellular regulation.

The first demonstration of an uptake pathway for apoptotic cells in mammalian systems was reported as a charge "sensitive" mechanism, inhibited by specific carbohydrates, and altered pH (Savill *et al.*, 1989a). Further investigation revealed this to be mediated by the integrin $\alpha_V\beta_3$ (Savill *et al.*, 1990), and the class B scavenger receptor, CD36, in human monocyte-derived macrophages (Savill *et al.*, 1992). $\alpha_V\beta_3$ and CD36 mediated binding of cell associated thrombospondin which was thought to act as a bridge or opsonin between the apoptotic cell and the phagocyte (Savill *et al.*, 1992). This mechanism was also found to function in murine bone marrow-derived macrophages (Fadok *et al.*, 1992b). In contrast murine peritoneal macrophages were demonstrated to predominantly use a novel receptor mediating recognition of externalised phosphatidylserine (Fadok *et al.*, 1992a), and it

was therefore proposed that macrophages from different tissues use distinct phagocytic pathways. In addition to the examples discussed above, Kupffer cells recognise altered carbohydrate residues on apoptotic particles (Dini *et al.*, 1995), whereas mouse thymic macrophages use class A scavenger receptors (Platt *et al.*, 1996). However murine bone marrow-derived macrophages were shown to switch to the utilisation of the PS system after stimulation, for example with digestible particles (Fadok *et al.*, 1993; Pradhan *et al.*, 1997). This suggests that the environmental stimuli a monocyte/macrophage receives at its origin may determine the molecular mechanism of uptake. Modulation of environmental stimuli are therefore likely to enable the promotion of specific phagocytic mechanisms. Understanding the influence of environmental factors on defective clearance in chronic inflammatory conditions may provide further opportunities for therapy.

Other putative receptors for macrophage clearance of apoptotic cells include those that also function as receptors of the innate immune system, CD14 and the mannose receptor. CD14 has been suggested to recognise an altered conformation of the adhesion molecule ICAM-3 (Devitt *et al.*, 1998; Flora and Gregory, 1994; Moffatt *et al.*, 1999), and the mannose receptor is thought to bind altered carbohydrate residues on the apoptotic cell surface (Dini, 1998; Hall *et al.*, 1994; Linehan *et al.*, 2000). Members of the scavenger receptor family also function in the innate immune system. They recognise a number of molecules including carbohydrate residues, lipids and bacterial LPS, and are able to mediate the uptake of particulate matter in addition to apoptotic cells. As the scavenger receptor family share homology with the only apoptotic cell receptor described in *C. elegans*, SREC (Zhou *et al.*, 2001), evolutionarily they may represent the oldest family of recognition receptors. During the development of the inflammatory response, other uptake mechanisms may have arisen from adaptation of pre-existing pathways in order to fulfil the huge requirement for clearance of inflammatory cells.

Intracellular mechanisms of phagocytosis

Inhibition studies using antibodies or competitive ligands to block specific receptor uptake never results in the complete ablation of apoptotic cell phagocytosis. For example, pharmacological inhibition of PS exposure cannot not reduce phagocytosis to that of control non-apoptotic populations (where limited phagocytosis and uptake still occurs) (Fadok *et al.*, 2001b). Furthermore, deletion of the class A scavenger receptor prevented phagocytosis of apoptotic cells *in vitro*, but had no effect on the cellular architecture of the thymus (Platt *et al.*, 1999; Platt *et al.*, 2000). The redundancy of receptor usage suggests that targeting the intracellular machinery mediating phagocytosis of apoptotic cells may provide an alternative focus for therapeutic intervention. The signalling pathways linking the extracellular receptor with the intracellular uptake machinery have not been fully explored, as research in this area is problematic. Primary cultures are the preferred choice of model system, however this restricts the ability to utilise molecular biology techniques for the expression of constitutively active or dominant negative mutants to assess the role of kinases, adaptor proteins or cytoskeletal components in phagocytosis. Additionally the apoptotic "meal" is highly heterogeneous due to the asynchronous progression of the apoptotic program within a cell population, and may present multiple "recognition" signals as discussed above. Furthermore engagement of each receptor type may activate a distinct intracellular pathway. The pathways thought to physically mediate apoptotic cell uptake share many common components not only with Fc- and complement phagocytosis mechanisms, but also regulation of adhesion. For example, cytoskeletal re-organisation during extension of membrane processes around the particle is thought to be regulated in a manner similar to that described during cell adhesion and migration. Work within the last few years has identified a major role for Rho-family GTPases in cytoskeletal organisation during cell adhesion and migration, and opsonized particle engulfment, providing a model for the intracellular regulation of apoptotic cell uptake (fig. 3).

Cytoskeletal regulation during adhesion and migration: Regulation of Rho GTPases

Rho GTPase proteins, a subgroup of the Ras superfamily, regulate a number of cellular functions including actin organisation and cell shape, cell adhesion and motility, membrane trafficking and gene expression (for review see Bishop and Hall, 2000). Rho GTPases are activated by ligation of cell surface adhesion receptors as well as by soluble mediators and chemokines. Rho proteins cycle between an inactive conformation when bound to GDP, and an active GTP-bound form. The GDP/GTP state is regulated by guanine-nucleotide exchange factors (GEFs) that promote dissociation of GDP and facilitate GTP binding. In contrast, guanine nucleotide dissociation inhibitors negatively regulate Rho protein signalling by preventing GDP/GTP exchange. A third level of regulation is mediated by GTPase activating proteins (GAPs) which stimulate the intrinsic GTPase activity of Rho proteins attenuating down stream signalling. A number of GTPase regulating proteins specific for Rho GTPases exist (Scita *et al.*, 2000). This redundancy may facilitate the differential localisation of Rho-GTPases within the cell for example lipid rafts (Tosello-Trampont *et al.*, 2001), in response to different extracellular stimuli allowing the induction of specific functional outcomes from a common signal (Zhou *et al.*, 1998).

Function of Rho-family GTPases during adhesion

Integrin ligation during adhesion to matrix activates src family kinases which mediate a number of intracellular signalling pathways culminating in the localisation and activation of Rho-family GTPases (fig 3) (for review see Schoenwaelder and Burridge, 1999) which regulate the formation of adhesion processes (Allen *et al.*, 1997; Rottner *et al.*, 1999). Podosomes are highly dynamic specialised adhesion structures found in cells of the monocyte lineage and in migratory cells where they are thought to play a role in maintaining cellular polarisation (see below). Podosomes are morphologically distinct from focal complexes characterised in immunofluorescence microscopy as punctate staining of integrins, structural proteins such as vimentin, vinculin, talin, adaptors including paxillin and p130cas, other

tyrosine phosphorylated proteins and substrates for protein kinase Cα (PKCα), assembled around an actin core (Marchisio *et al.*, 1984; Marchisio *et al.*, 1987; Nakamura *et al.*, 1993). Rho, Rac and Cdc42 have all been linked to the regulation of podosome formation and turnover (Burns *et al.*, 2001; Chellaiah *et al.*, 2000; Linder *et al.*, 1999; Ory *et al.*, 2000). The mechanisms governing this regulation have been most extensively characterised for Cdc42. Cdc42 is linked to the cytoskeleton via interaction with WASp (Wiskott-Aldrich Syndrome protein) (Aspenstrom *et al.*, 1996; Kolluri *et al.*, 1996; Symons *et al.*, 1996). WASp was first identified from Wiskott-Aldrich Syndrome patients where mutations in WASp resulted in severe immunodeficiency due to defects in leukocyte phagocytosis and migration (Kirchhausen, 1998; Kirchhausen and Rosen, 1996). Since its discovery a number of homologous proteins have been identified which also interact with Rho family GTPases mediating cytoskeletal rearrangement during adhesion and migration (for review see Takenawa and Miki, 2001). WASp interacts with the Arp2/3 complex of small G-proteins promoting actin polymerisation (Burns *et al.*, 2001; Linder *et al.*, 2000; Linder *et al.*, 1999; Machesky and Insall, 1998; Machesky *et al.*, 1999). Cells of the monocyte lineage derived from WAS patients lack podosomes. Additionally overexpression of inhibitory fragments of WASp disrupts podosome formation (Linder *et al.*, 2000; Linder *et al.*, 1999).

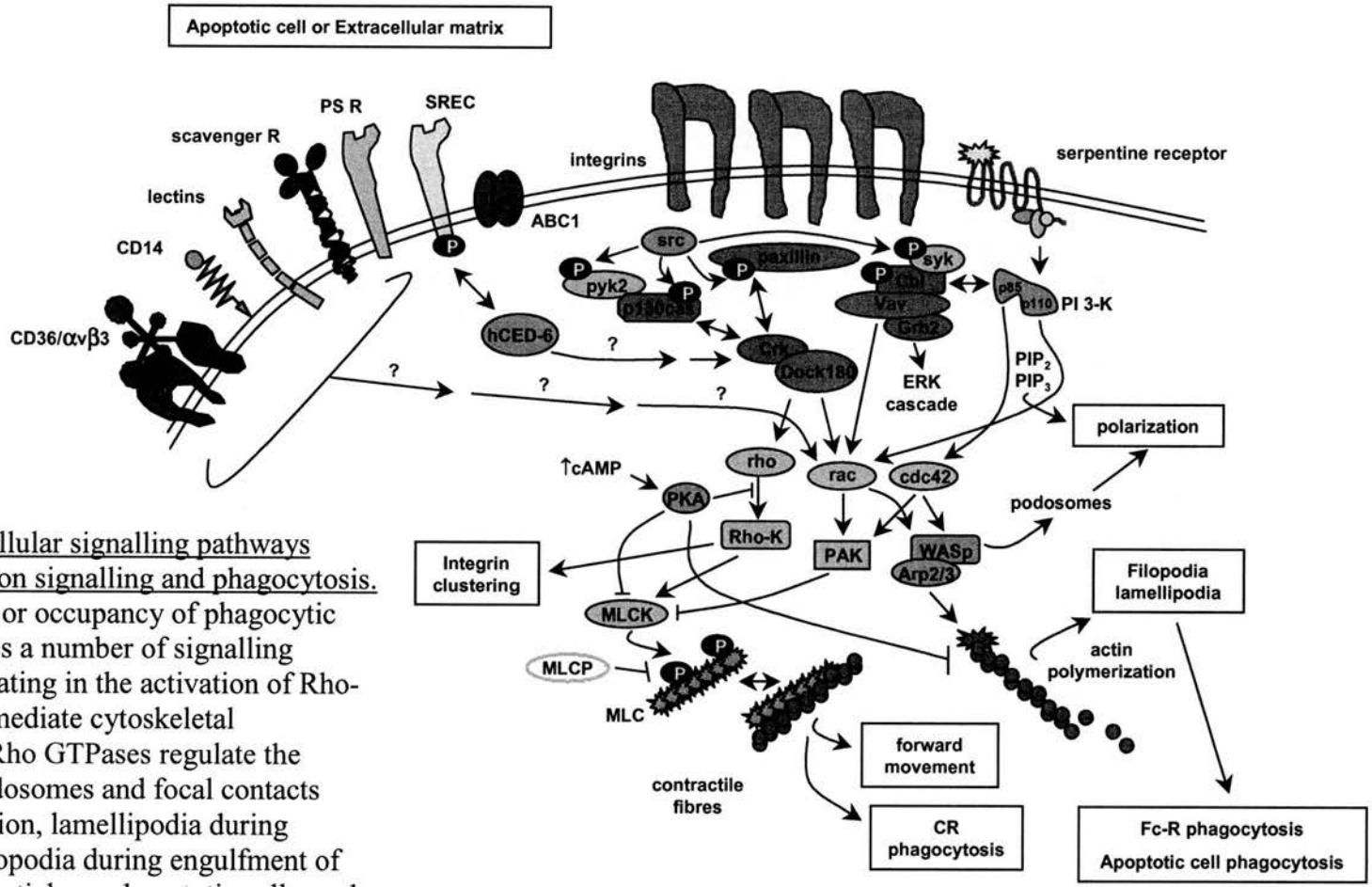


Figure 3. Intracellular signalling pathways mediating adhesion signalling and phagocytosis. Integrin ligation, or occupancy of phagocytic receptors, initiates a number of signalling pathways culminating in the activation of Rho-GTPases which mediate cytoskeletal reorganisation. Rho GTPases regulate the formation of: podosomes and focal contacts during cell adhesion, lamellipodia during migration, pseudopodia during engulfment of IgG-opsonized particles and apototic cells, and cellular contraction during cell movement and ingestion of complement opsonized particles.

Function of Rho GTPases during migration

During cell movement Cdc42 initiates the extension of short cellular protrusions, termed filopodia, supported by a core of bundled actin filaments (microfilaments). Filopodia have been observed in a number of cell types including macrophages and fibroblasts. Suspended between filopodia, are sheets of membrane enclosed cytoplasm, termed lamellipodia. These processes contain a network of myosin II-associated microfilaments required for cellular contraction during movement. Extension of filopodia and lamellipodia processes is mediated by actin polymerisation under the control of Cdc42 and Rac respectively. (Allen *et al.*, 1997; Kozma *et al.*, 1995; Nobes and Hall, 1995). Several different mechanisms for the initiation of actin polymerisation have been proposed (for review see Borisy and Svitkina, 2000). Severing and uncapping actin filaments exposes free barbed ends to which new actin subunits can be added. Alternately forward movement may be propelled by the *de novo* nucleation of actin polymerisation induced by the Arp2/3 complex and WASp family members. In filopodia, actin filaments are polymerised in a parallel fashion, in contrast to lamellipodia where the actin filament network is branched with Arp2/3 initiating formation of new filaments from the side of old ones (Borisy and Svitkina, 2000; Machesky and Gould, 1999). Branching is thought to contribute to the contractile capacity of lamellipodia during cell movement. During extension of adhesive processes, adhesive contacts and podosomes are formed at the leading edge of the cell to stabilise the membrane structures. These cell-ECM contacts also function as a base for contraction of cytoplasmic actomyosin filaments assembled in response to myosin light chain phosphorylation (Adelstein, 1983; Burridge and Chrzanowska-Wodnicka, 1996; Yoshioka *et al.*, 1998). Then with the release of contact sites in the trailing edge of the cell, mediated by RhoA (Alblas *et al.*, 2001; Worthylake *et al.*, 2001), forward movement occurs.

Fc Receptor-mediated phagocytosis

Particles opsonized by immunoglobulin G (IgG) are recognised by the Fcγ Receptor family. Particle binding for both IgG- and complement- opsonized particles does not

require cytoskeletal integrity, as attachment in the absence of intact microtubules and microfilaments has been observed (Caron and Hall, 1998; Newman *et al.*, 1991), and can occur at 4°C. Binding induces receptor aggregation, and intracellular signalling leading to the accumulation of a number of enzymes and adaptor proteins around the engaged particle. Actin, talin, paxillin and α-actinin, tyrosine phosphorylated proteins, and PKCα are enriched near to the phagosome membrane in a diffuse manner (Allen and Aderem, 1996; Allen and Aderem, 1995; Greenberg *et al.*, 1990; May *et al.*, 2000; Zheleznyak and Brown, 1992). Additionally a tyrosine kinase of the src family is thought to be activated upon FcR-aggregation, which phosphorylates residues in the FcγR cytoplasmic tail, termed ITAMs (immunoreceptor tyrosine based activation motifs) (Cox *et al.*, 1996). This allows the recruitment of the tyrosine kinase syk which triggers multiple pathways leading to inflammatory mediator release, gene expression and actin cytoskeleton rearrangement (Daeron, 1997; Daeron *et al.*, 1995). Macrophages from syk -/- mice show defects in particle ingestion but are still able to extend membrane processes, suggesting that syk may not be directly involved in actin polymerisation but in the completion of internalisation (Crowley *et al.*, 1997). FcR-mediated phagocytosis is unsurprisingly inhibited by compounds that perturb actin polymerisation dynamics, and tyrosine phosphorylation (Allen and Aderem, 1996; Greenberg *et al.*, 1993).

PI-3 kinase activity has also been shown to be required for FcR-mediated phagocytosis. Blockade of kinase function with pharmacological agents prevents phagosome closure and internalisation of IgG opsonized erythrocytes, without disturbing actin polymerisation at the point of particle attachment (Araki *et al.*, 1996; Cox *et al.*, 1999). The lipid products of PI-3 kinase are thought to regulate membrane recycling at the leading edge of cells during migration (Bretscher and Aguado-Velasco, 1998). Additionally, inhibition of recycling endosomes with tetanus toxin, an inhibitor of PI-3 kinase, inhibits phagocytosis (Araki *et al.*, 1996; Bajno *et al.*, 2000; Cox *et al.*, 1999; Ninomiya *et al.*, 1994).

Actin polymerisation and membrane extension allow the formation of cellular processes around the particle in a "zipper"-like fashion. Blockade of surface

receptors after particle attachment prevents engulfment (Griffin *et al.*, 1975). As discussed for cell migration above, co-ordination of actin polymerisation is thought to be mediated by Rho family GTPases that co-localise with the nascent phagosome (Caron and Hall, 1998). Treatment of phagocytic cells with toxin B from *Clostridium difficile*, an inhibitor of all Rho family members inhibits FcR-mediated phagocytosis without affecting particle recognition (Caron and Hall, 1998; Massol *et al.*, 1998). Specific inhibition of Rho with C3 transferase, another *Clostridium* toxin which ADP-ribosylates RhoA, suggests a role in receptor clustering required for the initiation of uptake (Caron and Hall, 1998). However, RhoA has been shown not to have a direct role in mediating cytoskeletal organisation during FcR-mediated phagocytosis (Massol *et al.*, 1998; May *et al.*, 2000). Microinjection of proteins, or protein expression constructs encoding dominant negative or constitutively active forms of Rac and Cdc42 have identified a role for these GTPases in FcR-mediated phagocytosis (Cox *et al.*, 1997; Massol *et al.*, 1998). Inhibition of Cdc42 appears to prevent pseudopod extension, whilst Rac1 is required for pseudopod fusion and phagosome closure. Recent work has also demonstrated a potential role for the Rho GEF Vav in mediating activation of Rac and Cdc24. Vav is tyrosine phosphorylated upon FcR clustering (Crespo *et al.*, 1997), and can be further activated by the lipid products of PI-3 kinase (Han *et al.*, 1998).

Rac and Cdc42 are thought to mediate actin rearrangement by engagement of WASp family members and the Arp2/3 complex, similar to the mechanisms employed during migration. Macrophages from WAS patients show deficiencies in phagocytosis, and GFP-tagged N-WASp has been shown to localise at the nascent phagosome (Zhang *et al.*, 1999). Furthermore, inhibition of Arp2/3 complex activation also abrogates engulfment (May *et al.*, 2000). Therefore we can describe a model whereby the zipper-like interactions of opsonins and FcRs, combined with a branched network of nucleating actin filaments, provides the force for pseudopod extension as for lamellipodia. Localised contractility has also been detected in the phagosome (Evans *et al.*, 1993). Both contractility and phagosome closure are sensitive to inhibitors of myosins which have also been detected associated with the nascent phagosome (Swanson *et al.*, 1999). Interestingly PAK-1, a serine/threonine

kinase activated by Cdc42 and Rac is also localised with the phagosome Dharmawardhane *et al.*, 1999). PAK-1 regulates contractility by inhibition of myosin light chain kinase (MLCK) (Sanders *et al.*, 1999). Closure of the phagosome is thought to require removal of the pre-assembled cellular scaffolding. Cytoskeletal and tyrosine phosphorylated proteins clustered at the site of ingestion rapidly dissociate as internalisation occurs (Greenberg *et al.*, 1991). This may be triggered by a negative feedback mechanism whereby phosphorylated proteins recruit tyrosine phosphatases via SH2 domain interactions, promoting de-phosphorylation and dispersion of localised proteins. Candidates include SHP-1 (Hunter and Avalos, 1998), and PSTPIP which associates with the PEST family of PTPases (Spencer *et al.*, 1997; Wu *et al.*, 1998).

Complement Receptor-mediated phagocytosis

Uptake of complement opsonized particles, although also dependent on cytoskeletal integrity, appears to proceed by an alternative mechanism. Particles sink into a phagocytic pit and are engulfed without the extension of pseudopodia (Kaplan, 1977). Cytoskeletal proteins such as paxillin, actin, vinculin and α-actinin are recruited to the membrane around the point of particle contact. Unlike Fc-mediated phagocytosis, these proteins are localised in a punctate fashion (Allen and Aderem, 1995; May *et al.*, 2000), and phagocytosis is not sensitive to inhibitors of tyrosine kinase activity, but do require PKC function (Allen and Aderem, 1995). PKC is thought to target the cytoplasmic portion of the β_2-integrin subunit, which may trigger receptor clustering observed upon particle engagement (Kwiatkowska and Sobota, 1999). Internalisation is thought to be induced by activation of RhoA with reorganisation of the actin cytoskeleton mediated by the Arp2/3 complex as found for Fc-mediated phagocytosis (Caron and Hall, 1998; May *et al.*, 2000). Introduction of dominant negative forms of Cdc42 or Rac have no effect on uptake by complement receptors (Caron and Hall, 1998; May *et al.*, 2000) and the link between RhoA and activation of Arp2/3 is not yet clear. The actin rich foci observed around complement opsonized particles are reminiscent of focal adhesions. During CR3-mediated uptake, RhoA signalling may promote clustering of integrins at the site of

ingestion (Dib *et al.*, 2001), in a manner that is analogous to its role during adhesion. Contractility generated against these sites of contact allow the particle to "sink" inside the macrophage.

Uptake of apoptotic cells

Studies to date demonstrate both similarities and differences between uptake of apoptotic cells, and FcR and CR-mediated phagocytosis. Engulfment of apoptotic cells requires actin polymerisation and cytoskeletal reorganisation, as for uptake of opsonized particles, and is likely to be more sensitive to cytoskeletal disruption due to the size differences between opsonized bacteria and apoptotic cells. Apoptotic cell binding requires intact cytoskeletal elements and our unpublished observations demonstrate tethering does not occur at low temperature. Studies of scavenger receptor function demonstrate binding of lipid microspheres is temperature insensitive (Bird *et al.*, 1999), such discrepancies are likely to be due to size constraints. Ingestion does not promote the release of pro-inflammatory cytokines as seen upon Fc-receptor mediated uptake, and actively down-regulates inflammatory processes by the release of anti-inflammatory cytokines (Fadok *et al.*, 1998a; Voll *et al.*, 1998). Immunofluorescent staining of macrophage-particle interactions revealed localisation of actin and tyrosine phosphorylated proteins in a diffuse manner similar to the Fc phagosome (Leverrier *et al.*, 2001; Leverrier and Ridley, 2001). Furthermore scanning electron microscopy revealed that engulfment proceeds by the extension of membrane processes around the particle similar to the "zipper" model of FcR uptake (Giles *et al.*, 2000; Leverrier and Ridley, 2001). Phagocytosis of apoptotic cells is predictably inhibited by agents that disrupt actin polymerisation and dynamics, but also pharmacological inhibitors of tyrosine kinases and PI-3 kinase (Leverrier and Ridley, 2001).

Prior to the initiation of this study an involvement of Rho-GTPases in phagocytic cell uptake was only hypothesised. However, experimental evidence within the last few years has unequivocally demonstrated a role for Rac in regulation of this cellular process. Elucidation of a putative uptake pathway was approached by two distinct

angles. The first was exploitation of the nematode worm *C.elegans* as a model system, the second was studies utilising molecular biology techniques for the overexpression or reconstitution of components of the hypothesised pathway using cell lines.

Phagocytosis in the nematode worm

During the development of the nematode worm, 131 of the 1090 somatic cells generated undergo programmed cell death (Sulston and Horvitz, 1977). Fourteen genes have been identified to be employed at various points of the apoptotic program, at least six of these function during engulfment and have homologues in the mammalian genome (for review see Hengartner, 2001). Genetic analysis of mutant worms has shown that the six engulfment genes are organised into two parallel pathways defined by the epistatic groups *ced-1, -7,* and *–8*, and *ced-2, -5, -10* and *– 12* (Chung *et al.*, 2000; Ellis *et al.*, 1991). The former pathway is less well characterised in both the worm and mammals. Cloning of *C.elegans ced-1* revealed it encoded a protein with homology to SREC a member of the scavenger receptor pathway (Zhou *et al.*, 2001), and is thought to bind apoptotic cells via exposed phosphatidylserine residues. The mammalian CED-7 counterpart is ABC1 (Wu and Horvitz, 1998a), a membrane protein of the ATP-binding cassette transporter family (for review see Broccardo *et al.*, 1999). Blockade of ABC1 function by pharmacological agents prevents phagocytosis (Luciani and Chimini., 1996), and its function has recently been reported to be required on both phagocyte and target due to its proposed ability for regulation of membrane phospholipid turnover (Hamon *et al.*, 2000; Marguet *et al.*, 1999). The *C.elegans gene ced-6* encodes an adaptor protein (Liu and Hengartner, 1998), its human homologue hCED-6 is also a cytosolic adaptor protein required for phagocytosis. It contains a number of protein interaction motifs, a phosphotyrosine binding (PTB) domain, a leucine zipper motif, and a proline rich region (Smits *et al.*, 1999; Su *et al.*, 2000). Overexpression of *ced-6* rescues *ced-1* and *–7* mutant worms placing the adaptor downstream in the pathway (Liu and Hengartner, 1998). Similarly overexpression of the mammalian homologue rescues the phenotype in the worm, and promotes engulfment in transfected J774

cells, a murine macrophage cell line (Liu and Hengartner, 1998; Smits *et al.*, 1999). Putative tyrosine phosphorylation motifs on the cytoplasmic tail of the newly characterised mammalian "CED-1" suggests that CED-1/CED-6 coupling may mediate the downstream signalling of this phagocytosis receptor.

Ced-2, -5, and –10 encode proteins homologous to the adaptor proteins Crk and DOCK180, and the Rho family GTPase Rac (Reddien *et al.*, 2001; Wu and Horvitz, 1998b). Formation of this complex is induced by integrin ligation. Interaction of Crk/CED-2 with DOCK180/CED-5 induced membrane localisation and activation of Rac/CED-10, resulting in actin polymerisation during membrane ruffling, adhesion and migration (Albert *et al.*, 2000; Kiyokawa *et al.*, 1998; Reddien *et al.*, 2001; Tosello-Trampont *et al.*, 2001). Human DOCK180 is able to rescue defects in cell migration in *ced-5* mutants, but not altered phagocytosis (Wu and Horvitz, 1998b), suggesting more components of this pathway in the mammalian system remain to be identified. Albert and co-workers and Tosello-Trampont and colleagues independently demonstrated a direct function for the crk/DOCK180/Rac pathway for phagocytosis in mammalian cells. Dendritic cells were found to utilise $\alpha_V\beta_5$ integrins for the recognition of apoptotic proteins, which engaged the crk/DOCK180/Rac complex via the adaptor protein p130cas (Albert *et al.*, 2000). Tosello-Trampont and colleagues similarly demonstrated the formation of the crk/DOCK180/rac complex upon particle engulfment (Tosello-Trampont *et al.*, 2001) . The link between Rho-GTPase activation and actin polymerisation is thought to be identical to that instigated during cell migration and adhesion, as macrophages from WAS patients show defects in phagocytosis of apoptotic cells (Leverrier *et al.*, 2001).

Regulation of macrophage phagocytosis of apoptotic cells

The redundancy in receptor usage for phagocytosis of apoptotic cells demonstrated by *in vitro* and *in vivo* studies suggests that therapeutic intervention at this level would be unlikely to promote engulfment further within macrophage populations. Targeted expression of phagocytic receptors in "non-professional" phagocytes could

augment the phagocytic capacity of specific tissues. However, this approach may be limited to the cytoskeletal components required for uptake of large particles. Opsonization of apoptotic cells in a tissue-specific manner may allow the upregulation of individual pathways in organs that show defective clearance. One potential problem is that the full extent of redundancy within this system is not yet fully clear. Regulation of the intracellular mechanisms that represent rate limiting events for phagocytosis are likely to have important therapeutic potential, but may represent a difficult target in terms of specificity. A number of exogenous factors have been shown to augment phagocytic clearance, including soluble mediators such as cytokines, ligation of adhesion receptors, and glucocorticoids, which may mediate their effects via modulation of intracellular pathways. Investigation into the mechanism of their action may allow the manipulation of phagocytic capacity.

Soluble mediators

During inflammation the tissue microenvironment contains elevated levels of specific cytokine and inflammatory mediators, that regulate apoptotic pathways in leukocytes (Lee *et al.*, 1993). It might be expected that these substances also affect phagocytic capacity. Incubation of human monocyte-derived macrophages with granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β, IFNγ, TNFα or TGFβ1 for four hours was found to augment macrophage phagocytosis of apoptotic neutrophils (Ren and Savill, 1995). However phagocytic capacity in other macrophage models has demonstrated an inhibitory effect of IFNγ upon uptake of apoptotic neutrophils (Erwig *et al.*, 1998), although this study corroborated the increase in phagocytosis following TNFα exposure. Such discrepancies may be due to the tissue origin or species of phagocytes used for the studies, the differentiation state of macrophages, or whether these cells have been exposed to cytokines and inflammatory mediators. Prior exposure of rat bone marrow derived macrophages to IFNγ prevented subsequent stimulation of apoptotic cell uptake by TNFα (Erwig *et al.*, 1998). More recently, glucocorticoids have been demonstrated to augment phagocytosis of apoptotic neutrophils by peripheral blood monocyte-derived

macrophages and murine bone-marrow derived macrophages. Endogenous glucocorticoids are released during inflammatory "stress" (Chrousos, 1995) and have been shown to have a number of immunomodulatory functions discussed further in the next section.

Adhesion signalling

Substances that elevate intracellular cyclic AMP (cAMP) and activation of PKA such as the inflammatory mediator prostaglandin E_2, have been shown to modulate granulocyte apoptosis (Chang *et al.*, 2000; Ottonello *et al.*, 1998; Peacock *et al.*, 1999; Rossi *et al.*, 1995) and macrophage phagocytic function (Rossi *et al.*, 1998). The disruption in particle uptake was attributed to the disruption of cytoskeletal elements and cell detachment that accompanied prostaglandin treatment. We have described how the components of the phagocytic machinery are common to those required for cytoskeletal organisation during migration and adhesion, therefore it is likely that the adhesion status of the cell may contribute to its phagocytic potential, directly by signals arising from integrin ligation (Albert *et al.*, 2000; Erwig *et al.*, 1999), or indirectly by adhesion modulating the response to cytokines and other soluble mediators (Nathan and Sporn, 1991). During the inflammatory response there is likely to be extensive remodelling of ECM which could function as a temporal stimulus for augmented phagocytosis (Gailit and Clark, 1994). Furthermore, with increased characterisation of macrophage-ECM interactions a growing number of specific examples of adhesion modulation of phagocytic potential may become obvious.

Cross-linking of the cell surface glycoprotein CD44 rapidly augments the phagocytosis of apoptotic neutrophils by human peripheral blood monocyte-derived macrophages (Hart *et al.*, 1997). CD44 is proposed to bind a number of ECM ligands including hyaluronan and fibronectin, and has been implicated in cell motility during metastasis (Lesley *et al.*, 1997; Lesley *et al.*, 1993). The intracellular region of CD44 is thought to interact with the Ezrin/radixin/moesin (ERM) family of

proteins which are able to mediate cytoskeletal organisation and signalling (Tsukita *et al.*, 1994). Previously identified inhibitors of apoptotic cell recognition were unable to block neutrophil uptake in response to CD44 ligation, suggesting either redundancy of receptor usage or the engagement of unique receptors. The latter represents an exciting possibility as CD44 ligation was unable to promote phagocytosis of other apoptotic particles (Hart *et al.*, 1997).

Integrin ligation has been strongly linked to both a positive and negative regulation of phagocytosis. Ligation of $\alpha_V\beta_3$, $\alpha_1\beta_2$ or $\alpha_6\beta_1$ during apoptotic cell uptake, or by mAb has been reported to inhibit subsequent phagocytosis when macrophages were re-challenged with apoptotic cells, 48 hrs later (Erwig *et al.*, 1999). The mechanism for this "programming" is not clear, integrins are able to activate a number of intracellular pathways leading to cytoskeletal rearrangement, release of soluble mediators and changes in gene expression (Aplin *et al.*, 1998), which could have effects on the subsequent phagocytic capacity. In contrast to studies by Erwig and co-workers, other research groups have demonstrated a positive function of β_1 and β_3 -integrins during phagocytosis. They have been described to mediate both apoptotic cell recognition (Albert *et al.*, 2000; Savill *et al.*, 1990; Schwartz *et al.*, 1999), and modulation of capacity when bound to ECM components such as fibronectin (McCutcheon *et al.*, 1998). Additionally the $\alpha_V\beta_5$ integrin complex has recently been shown to be linked to activation of Rac, required for phagocytosis of apoptotic cells by dendritic cells (Albert *et al.*, 2000).

In addition to integrins, several macrophage "phagocytic receptors" have also been described to function during cell adhesion. The scavenger receptor-A has been shown to be involved in divalent cation-independent adhesion to serum-coated cell culture plastic (Fraser *et al.*, 1993). The adhesive role of scavenger receptors appears to be augmented following macrophage activation, suggesting SR-A may play a role in the retention of macrophages at the site of inflammation (van Velzen *et al.*, 1999), which may indirectly promote clearance of apoptotic cells. Furthermore, characterisation of an involvement of scavengers receptors in the formation of atherosclerotic plaques suggests a potential role for scavenger receptors in

phagocyte-endothelial interactions (Maxeiner *et al.*, 1998). CD36, a class B scavenger receptor can mediate adherence to surfaces coated in collagen, and certain anti-CD36 mAbs mimic the anti-adhesive effects of the ECM molecule thrombospondin (Dawson *et al.*, 1997). CD36 may also associate with the tyrosine kinases fyn, yes, and lyn (Huang *et al.*, 1991) to enable mediation of adhesion signalling. Interestingly activation of src family kinases fgr, hck and lck upon integrin ligation has been linked to the modulation of cytoskeletal organisation. Peritoneal exudate macrophages from $fgr^{-/-}$, $hck^{-/-}$ and $lck^{-/-}$ mice show defects in migration and phagocytosis of IgG opsonized particles (Suen *et al.*, 1999).

Glucocorticoid modulation of inflammation

Glucocorticoids have been widely used for the treatment of immune and inflammatory diseases due to their immunomodulatory effects affecting T cell activation, adhesion molecule expression, cell migration, effector cell activation and cytokine production (table 3) (Schleimer, 1993; Wilckens and De Rijk, 1997). Endogenous glucocorticoids are secreted by the adrenal glands in response to stress including inflammation (Chrousos, 1995). Circulating cytokines including IL-1, IL-6 and TNFα directly stimulate the hypothalamus and/or the pituitary, resulting ultimately in the release of cortisol, which in turn is inhibitory to the expression of these cytokines (Chrousos, 1995). Endogenous glucocorticoids are therefore thought to function in this capacity to promote the down-regulation and resolution of inflammatory processes.

Glucocorticoids exert their effects by binding to glucocorticoid receptors localised in the cytoplasm of target cells. Two isoforms of the glucocorticoid receptor exist, α and β, with the signalling defective β-form acting as an antagonist to glucocorticoid stimulation (Bamberger *et al.*, 1995; Oakley *et al.*, 1999; Strickland *et al.*, 2001). The glucocorticoid receptor consists of a ligand binding domain at its C-terminus, a central DNA binding region composed of two zinc finger motifs, plus an N-terminal domain involved in the transcriptional trans-activation of genes and interaction with other transcription factors (for review see Whitfield, 1999). The unoccupied receptor

is bound to a multi-protein complex composed of two molecules of hsp90, a heatshock protein, in addition to immunophilin, a chaperone molecule, which prevents unoccupied GR localising to the nuclear compartment. Upon glucocorticoid binding, hsp90 dissociates revealing two nuclear localisation signals at the C-terminus of the receptor promoting translocation to the nucleus and DNA binding. In the nucleus the receptor usually binds as a homodimer to glucocorticoid response elements (GREs) (Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991), although dimerisation has been shown not to be required for all trans-repressional activity (Reichardt *et al.*, 1998). GREs are composed of highly conserved palindromic 15 base pair DNA elements. Negative GREs (nGREs) which confer GC-dependent repression upon target genes display a more variable DNA sequence (Drouin *et al.*, 1993). Transcriptional effects of the glucocorticoid receptor are not confined to activation via GREs. The receptor/hormone complex is able to affect transcription of genes which contain no GREs or nGREs via repression of other transcription factors such as AP1, STAT5, and NF-κB, with the resulting inhibition of a number of genes (table 4) (Jenkins *et al.*, 2001).

Recently glucocorticoids have been shown to specifically augment phagocytosis of apoptotic cells, but not opsonized particles (Liu *et al.*, 1999). The augmented ingestion was non-phlogistic and suggests another mechanism whereby glucocorticoids exert their anti-inflammatory functions. However, long term steroid use is associated with a number of undesirable side effects which include retention of sodium and water, excretion of potassium, osteoporosis, hypertension, diabetes, gastric ulcers and skin atrophy (Boumpas, 1993). Elucidation of the mechanism of glucocorticoid-augmented phagocytosis would allow the development of dissociated steroids that may limit the induction of side effects. The previous studies characterised the effect of short term (24hr) glucocorticoid exposure of monocyte/macrophages. Preliminary studies suggested that longer term treatment (up to 5 days) further increased phagocytic capacity, and would be more analogous to *in vivo* exposure of monocyte/macrophages in patients on steroid therapy.

Cell type	Effect
Inflammatory cells	
Eosinophil	↑ apoptosis ↓ release from bone marrow and recruitment to tissues ↓ inflammatory mediator production
Neutrophil	↑ viability
Mast Cell	↑ Apoptosis
T lymphocyte	↓ cytokine releases ↓ recruitment
Dendritic cell	↓ maturation
Macrophage	↓ cytokine production ↓ recruitment

Table 3. Effect of glucocorticoids on inflammatory cells.
Adapted from Barnes, 1997.

Increased transcription	Decreased Transcription
Lipcortin-1	Cytokines: IL-1, -2, -3, -4, -5, -6, -8, -11, -12, -13, TNFα,
β_2-adrenoreceptor	GM-CSF, RANTES, MIP1α)
Secretory leukocyte inhibitory protein	Inducible nitric oxide (iNOS)
IκB-α	Inducible cyclooxygenase (COX-2)
	Inducible phospholipase A_2 (PLA_2)
	Endothelin-1
	NK_1-receptors
	Adhesion molecules (ICAM-1, VCAM-1)

Table 4 Effects of glucocorticoids on gene transcription in inflammation
Adapted from Barnes, 1997.

Aims

The primary aims of this study were to investigate the mechanism of phagocytosis of apoptotic cells, principally by characterising pathways involved in augmented clearance of apoptotic cells elicited by long-term glucocorticoid treatment of peripheral blood monocyte-derived macrophages during *in vitro* culture. When these studies were initiated, the regulation of cytoplasmic modulators of phagocytosis of apoptotic cells was largely uncharacterised. Experimental evidence concerning the uptake of IgG and complement opsonized particles suggested a role for adhesion and adhesion signalling components in the regulation of these processes. Glucocorticoids have been shown to alter dendritic cell maturation, and exert immunomodulatory effects on macrophage function, including induction of alternatively activated cells. Considering the central role of apoptotic cell clearance in resolution processes we also sought to assess the effect of glucocorticoids on macrophage differentiation and the potential wider ranging effects on immune function. Specifically we asked:

1. What were the effects of glucocorticoid treatment on putative phagocytic receptor expression and function?

2. What were the effects of glucocorticoid treatment on macrophage adhesion and cytoskeletal organisation?

3. How do glucocorticoids influence monocyte differentiation *in vitro* and the responses to polarising immune cytokines.

CHAPTER 2: MATERIALS AND METHODS

Antibodies and other reagents

Reagents were obtained from Sigma (Poole, UK) unless otherwise stated. Iscove's DMEM (IDMEM) was from Gibco BRL (Paisley, UK). Dextran and Percoll™ were from Amersham Pharmacia Biotech, (Buckingham, UK). Dexamethasone was obtained from David Bull Laboratories (Warwick, UK). Primary antibodies were from the following sources: p130cas, paxillin, Pyk2, Rac and RC-20 (anti-phosphotyrosine) mAb were from Transduction Laboratories (supplied by Becton-Dickinson, Oxford, UK). Mannose receptor was from Pharmingen (supplied by Becton-Dickinson, Oxford, UK). HA monoclonal, CrkL and C3G rabbit polyclonal antibodies were from Santa Cruz (supplied by Insight Biotechnology, Wembley, UK). mAb specific for CD44v3 (3G5), β1 (12G10) and class II (WR18) were from Serotec (Oxford, UK). Control mouse immunoglobulins (IgG1and IgG2a), BerMac3 (CD163) and rabbit immunoglobulins, and $F(ab')_2$ goat anti-mouse immunoglobulin FITC and HRP conjugates were from DAKO (Cambridge, UK). The following monoclonals were generously provided as gifts: 5A4 (CD44 - Dr. Graeme Dougherty, UCSF, CA), smϕ and 15.2 (CD36 and CD54 respectively - Dr. Nancy Hogg, ICRF, London), 23C6 (CD51/61 - Dr. Mike Horton, UCL, London), PM6/13 (CD61 – Dr. Mike Wilkinson, Wellcome Trust, UK), 3G8 (CD16 - Dr. Jay Unkeless, Mount Sinai Medical School, New York), UCHM1 (CD14 – Dr Peter Beverley, UCL, London), 61D3 (CD14 – Prof. Chris Gregory, Nottingham, UK). Secondary anti-rabbit HRP was from Amersham Pharmacia Biotech. Goat anti-mouse and goat anti-rabbit-Alexa™ conjugated secondary antibodies were supplied by Molecular Probes (Oregon, USA).

Cell Isolation

Mononuclear and polymorphonuclear leukocytes were isolated as previously described (Hart *et al.*, 1997). In brief, freshly drawn peripheral blood from healthy volunteers (typically 160ml) was collected into sodium citrate (Phoenix

Pharmaceuticals Ltd., Gloucester, UK) (final concentration 0.38%) into 50ml Falcon tubes, and centrifuged at 350 x *g*, for 20 min, resulting in an upper fraction (platelet-rich plasma) which was removed for preparation of autologous serum (see *Cell culture*) and a lower cell pellet. Erythrocytes were removed by sedimentation with 0.6% (w/v) dextran T500 (2.5ml dextran/10ml cell pellet, made up to 50ml total volume with saline) for approximately 30 minutes at room temperature. The upper leukocyte rich layer was then removed, made up to 50 ml with saline to remove excess dextran and centrifuged at 350 x *g*, for 6 min. Leukocytes were then fractionated on a discontinuous Percoll™ gradient (two gradients sufficient to separate up to 160ml of blood). Percoll was made isotonic with 10X PBS (90% solution) and gradients prepared by overlaying 2.5ml of each Percoll 73%/63%/50% gradient. Bottom layer 73%: 4.05ml 90% Percoll, 0.95ml PBS (w/o Ca^{2+}/Mg^{2+}); middle layer 63%: 3.5ml 90% Percoll, 1.5ml PBS (w/o Ca^{2+}/Mg^{2+}); bottom layer: pelleted cells resuspended in 2.5ml 50%: 2.75ml 90% Percoll, 2.25ml PBS (w/o Ca^{2+}/Mg^{2+}), then centrifugation at 720 x *g* for 20 min at room temperature. MNC were aspirated from the 50/63% interface, and PMN from the 63/73% interface, with residual erythrocytes pelleted at the bottom. Leukocytes were washed three times in PBS (w/o Ca^{2+}/Mg^{2+}) before culture, and assessment of purity and neutrophil activation status (see *Quality control*)

Cell Culture

Neutrophils were resuspended at $4x10^6$cells/ml in IDMEM containing 10% autologous serum (prepared by re-calcification of platelet rich plasma, 220µl of 1M $CaCl_2$/10ml plasma, final concentration 22mM) and cultured at 37°C in a 5% CO_2 atmosphere for 20h in Falcon tissue culture flasks. Alternatively neutrophils were resuspended in IDMEM at $20x10^6$/ml and incubated with 2µg/ml (final concentration) CellTracker™ Green 5-chloromethylfluorescein diacetate (Molecular Probes, Oregon, USA), 20 min, 37°C; then incubated for 20h in autologous serum as before.

MNC were plated at $4x10^6$/ml in IDMEM and incubated for 30-60 min, at 37°C, 5% CO_2, after which non adherent lymphocytes were removed by washing 3-4 times with HBSS (without Ca^{2+}/Mg^{2+}). The resulting monocyte monolayer was cultured for a period of five days in IDMEM plus 10% autologous serum, ± 1μM dexamethasone. The resulting cells showed increased expression of CD14, CD16, and the $\alpha_V\beta_3$ integrin, and were negative for myeloperoxidase activity, characteristic of a macrophage phenotype.

Quality control

Mononuclear cell and polymorphonuclear cell preparations were assessed for purity by flow cytometry. Figure 1 shows the light scatter profiles of mononuclear, and polymorphonuclear cell preparations using either an EPICS Profile II (Beckman-Coulter, High Wycombe, UK) or a FACScaliber (Becton-Dickinson, Oxford, UK) flow cytometer. Mononuclear and polymorphonuclear cell preperations were routinely above 95% purity.

Characterisation of neutrophils apoptosis

After culture for 20h in autologous serum, neutrophil populations were >50% apoptotic as determined by morphological analysis and Annexin V binding, and <5% necrotic as defined by propidium iodide exclusion (fig. 2). Neutrophils ($4x10^6$/ml) were cytocentrifuged at 300rpm, 3min, fixed with 100% methanol and visualised with Diff-Quick™ staining and 100x oil immersion light microscopy. Viable cells contained the characteristic neutrophil multi-lobed nuclear morphology compared to the darkly stained pyknotic nuclei of apoptotic cells (Kerr *et al.*, 1972).

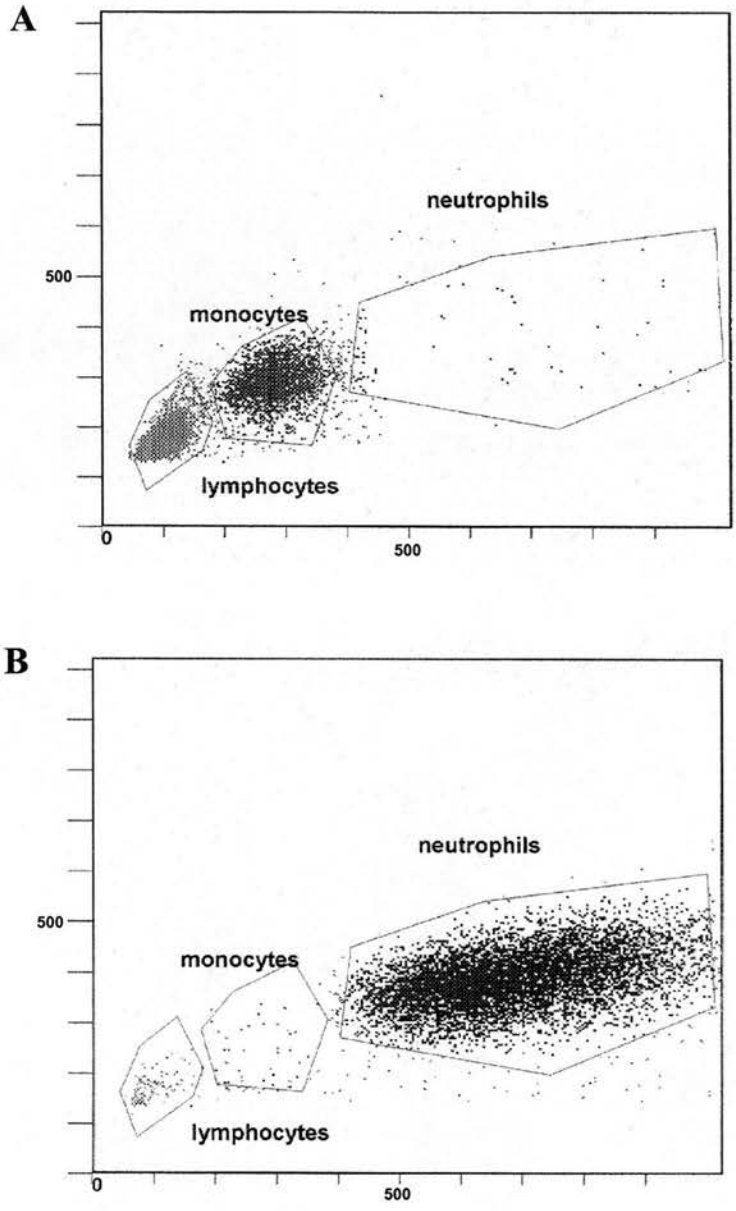


Figure 1. Purity of leukocyte preparations
Mononuclear and polymorphonuclear cells were isolated from peripheral blood via dextran sedimentation followed by separation on a discontinuous Percoll™ gradient. Laser scatter properties of cell populations were assessed by flow cytometry.
A mononuclear cell fraction. **B** Polymorphonuclear fraction. Purity ratios for panel A, monocytes: 32.6%, lymphocytes: 62.9%, neutrophils: 1.1%; for panel B, monocytes 0.5%, lymphocytes 1.4%, neutrophils 94.8%.

Annexin V binding and PI staining

190µl of a 1:500 dilution of Annexin V-FITC was added to 10µl of neutrophil suspension, incubated for 10 min, room temperature. 1µl of 1mg/ml PI was added to the cell/Annexin V mix and immediately analysed by flow cytometry for Annexin V binding on FL-1 and PI exclusion on FL-2.

Macrophage Phagocytosis assay

Monocyte-derived macrophages were cultured in 48 well tissue culture plates as described above for five days in the presence or absence of DX, or 10µM RU38486 for varying periods of time. Apoptotic neutrophils were washed once in IDMEM to remove serum and resuspended in IDMEM at $4x10^6$/ml. Macrophage monolayers were washed once in IDMEM then overlaid with a "lawn" of apoptotic neutrophils, and incubated at 37°C, 5% CO_2, for 20 minutes.

Plate based assay

After the incubation at 37°C, ingested neutrophils were removed by washing 2-3 times with IDMEM. Monolayers were then fixed in 2.5% glutaraldehyde (in PBS), 20 min, room temperature, after which ingested neutrophils were visualised by staining for myeloperoxidase activity with 0.1mg/ml dimethoxybenzidine (in H_2O) and 0.03% (v/v) hydrogen peroxide (20 min, room temperature). The percentage of macrophage phagocytosis was quantified microscopically by counting at least 500 cells per well from randomly selected fields, and an average between the duplicate wells calculated (fig 3). Phagocytic index was calculated as (average number of neutrophils phagocytosed per macrophage) x (% macrophages that had phagocytosed one or more neutrophil).

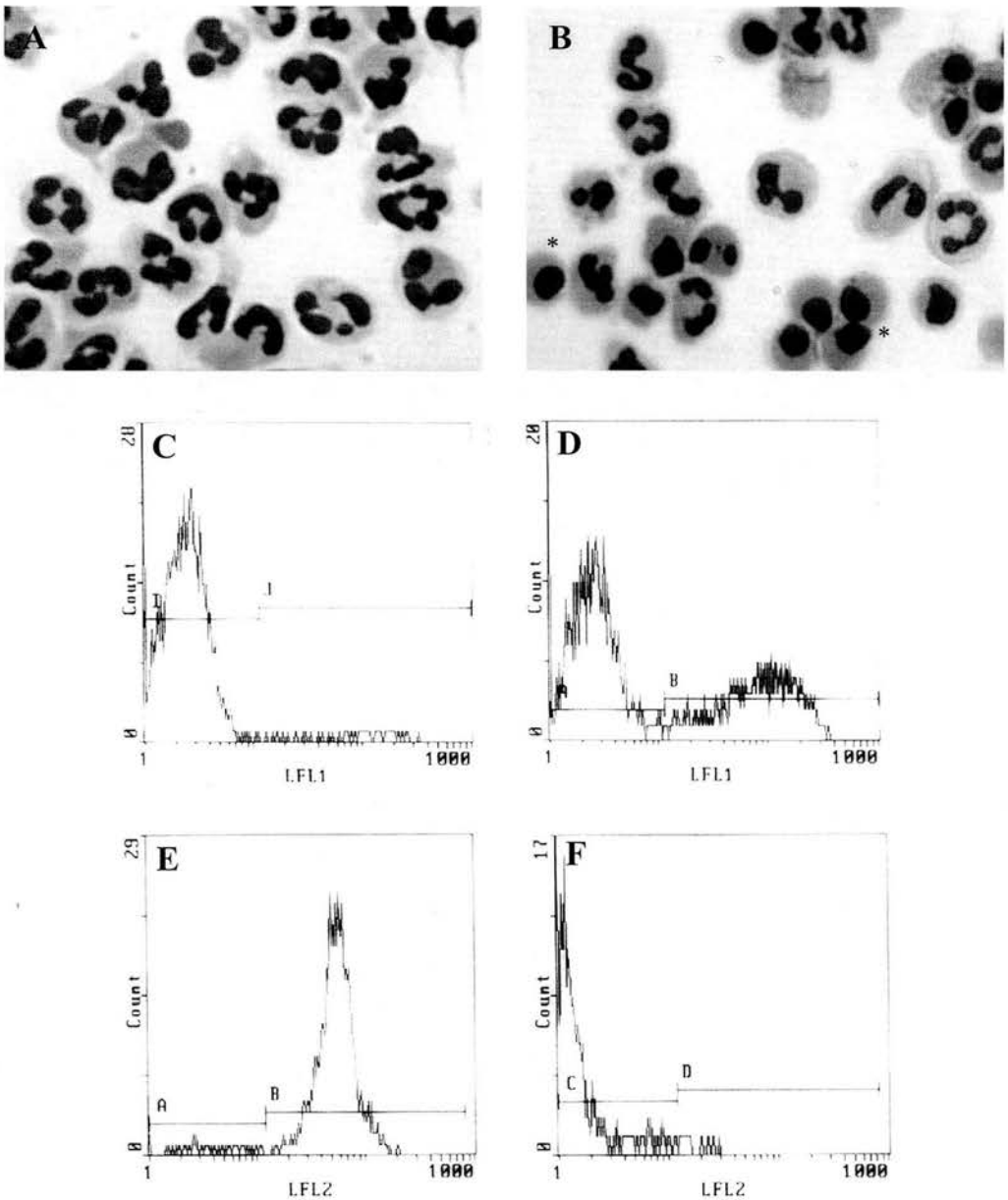


Figure 2. Assessment of Neutrophil apoptosis

Peripheral blood neutrophils were cultured for 20 hr in IDMEM containing 10% autologous serum. Neutrophil viability was determined by assessment of morphological characteristics in cytocentrifuge preparations, and flow cytometric analysis of Annexin V binding and propidium iodide exclusion. **A** Freshly isolated neutrophils, note the high percentage of viable cells containing multi-lobed nuclei. **B** Aged neutrophils, apoptotic cells are characterised by the presence of pyknotic nuclei (condensed rounded), indicated by *. Flow cytometric profiles of Annexin V binding. **C** Viable cells demonstrate low AnnexinV binding. **D** Aged neutrophils, approximately 55% Annexin V positive. **E** Necrotic neutrophils generated by incubation at 70°C, 10 minutes, populations were 98% positive for propidium iodide inclusion. **F** Aged, non-necrotic neutrophils, 97% propidium iodide negative.

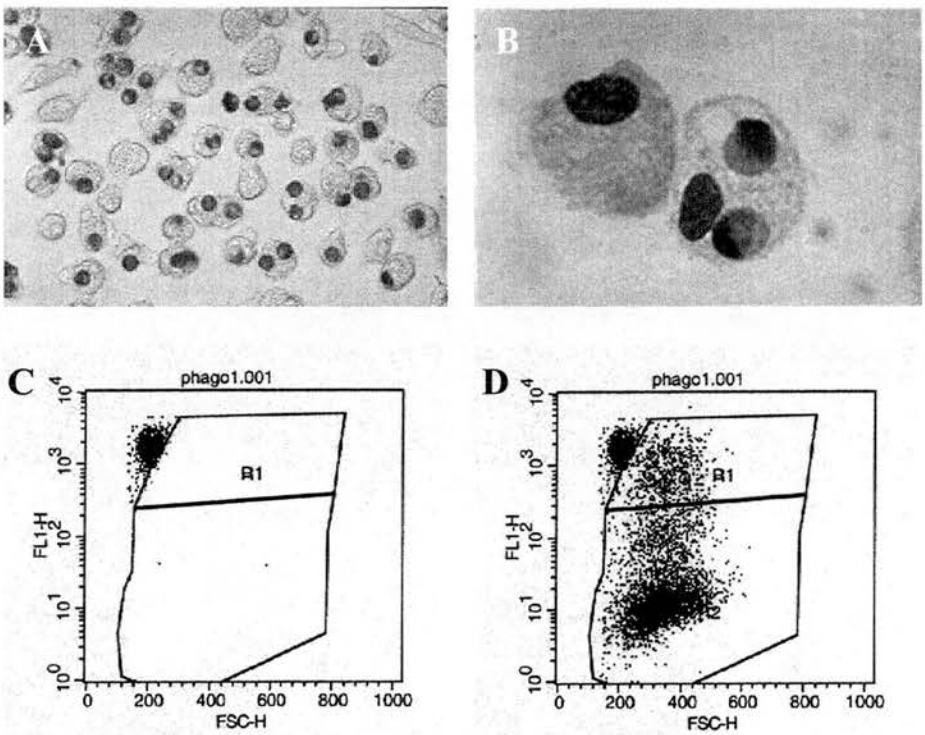


Figure 3. Quantitation of macrophage phagocytosis of apoptotic neutrophils
Following incubation with apoptotic neutrophils the percentage of cells ingesting apoptotic particles was assessed. **A** Ingested neutrophils were visualised by staining for myeloperoxidase activity, followed by microscopic analysis (Panel shows phagocytosis after glucocorticoid stimulation). **B** Cytocentrifuge preparation demonstrating internalisation of apoptotic cells. **C** Flow cytometric analysis of cell-tracker green^{TM} fluorescently labelled aged neutrophils. **D** Macrophages ingesting cell-tracker green^{TM} labelled neutrophils. Gate R1 corresponds to macrophages demonstrating high fluorescence and therefore uptake of neutrophils. Note the population of macrophages with intermediate levels of fluorescence, which is thought to represent uptake of cellular fragments not visible under normal light microscopy.

Flow cytometry assay

After incubation at 37°C, media was aspirated from wells directly and replaced with 500µl of Trypsin-EDTA. Cells were then incubated at 37°C for 20 min, then 4°C for 30min, followed by rigorous pipetting on ice to ensure removal of all adherent cells. The percentage macrophages phagocytosing apoptotic particles was assessed by flow cytometry. Macrophage populations were gated on forward and side scatter characteristics, and the percentage of cells within this population demonstrating green fluorescence due to the uptake of labelled neutrophils was calculated (fig 3). Double staining for neutrophil markers prior to flow cytometric analysis demonstrated that trypsinisation was sufficient to remove neutrophils bound to macrophages and the green fluorescence corresponded solely to uptake of fluorescent particles (H. Jersmann *et al.*, manuscript in preparation).

Assessment of CD44 augmented phagocytosis of apoptotic neutrophils

Macrophages were washed once with IDMEM, then incubated for 15 min at 37°C, 5% CO_2 with CD44 mAb 5A4 (1:5 dilution of hybridoma supernatant). Cells were washed twice to remove antibody prior to addition of apoptotic cells as described above.

Inhibition of macrophage phagocytosis

Macrophages were washed once with IDMEM, then incubated for 15 min at 37°C, 5% CO_2 with phagocytosis inhibitors (RGDS/RADS, 0.5mM; smϕ, 1:50 ascites; glucosamine, 10mM; mannan, 200µg/ml; My4, 10µg/ml; phospho-L-serine, 2mM; dextran sulfate, 200µg/ml; fucoidan, 500µg/ml; glyburide, 100µM). Apoptotic cells were then added to a final concentration of $4x10^6$/ml without removal of inhibitor (except for smϕ and My4 antibodies), and assessed as for the plate based assay.

Controls for inhibitors

All mAb used have previously been shown to inhibit apoptotic cell phagocytosis and were used at concentrations that were deemed to be saturating by flow cytometry.

The following inhibitors were found to be functionally active at concentrations used in this study. RGDS (integrin inhibition peptide) was shown to inhibit $\alpha 5\beta 1$ integrin mediated adhesion of T lymphocytes to fibronectin. Phospho-L-serine was shown to inhibit binding of FITC-conjugated Annexin V to apoptotic neutrophils in flow cytometric analysis. Concentrations required to inhibit Annexin V binding were higher than that used for inhibition of apoptotic cell uptake due to the high binding affinity of Annexin V for PS residues compared to macrophage cell surface receptors. (fig. 4a). Dextran sulfate and fucoidan inhibited uptake of acetylated-LDL by monocyte-derived macrophages (fig. 4b). Other reagents (glyburide, mannan, and glucosamine) were used at concentrations that have previously been shown to exert inhibitory effects.

Flow cytometry

Flow cytometry was performed essentially as described (Dransfield *et al.*, 1994). Monocyte-derived macrophages were cultured for 5 days ± 1μM DX in 6-well tissue culture plates as described above. Cells were washed once in PBS (w/o Ca^{2+}/Mg^{2+}) then incubated on ice in 2.5mM EDTA plus 0.5% serum (v/v) in PBS (w/o Ca^{2+}/Mg^{2+}) for 30-40 min, followed by pipetting to dislodge adherent cells. Cells were then washed with ice cold PBS containing 0.2% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide (PBN), to remove EDTA, and 10^5/cells/assay pelleted in 96-well flexiwell plates by spinning at 300 x *g* for 3 min at 4°C. All subsequent incubations were carried out on ice to prevent internalisation of antibody. Cells were resuspended and pre-incubated for 10 minutes with 20% (v/v) normal rabbit serum to block non-specific binding to Fcγ receptors. Cells were then incubated with saturating concentrations of mAb for 30 minutes and washed twice in PBN prior to incubation with FITC-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin (DAKO)

(1:40), for 30 min and washed twice more before analysis using either an EPICS Profile II (Beckman-Coulter, High Wycombe, UK) or a FACScaliber (Becton-Dickinson, Oxford, UK) flow cytometer.

Electron Microscopy

Macrophages cultured on glass coverslips in the presence or absence of 1μM dexamethasone for 5 days were fixed with 3% gluteraldehyde in 0.1M sodium cacodylate buffer pH7.4 for 3h, and post fixed with 1% osmium tetroxide in 0.1M cacodylate buffer for 2h. After dehydration in an ascending acetone series, and critical point drying with CO_2, samples were sputter coated with 20nm gold/palladium and examined using a Phillips 505 scanning electron microscope.

Immunofluorescence

Monocyte-derived macrophages were cultured on glass cover slips, in 12-well tissue culture plates for 5 days ± 1μM DX as described above. Cells were washed once with IDMEM to remove serum proteins, then fixed in 3% paraformaldehyde (PFA) in PBS, for a minimum of 20 min at room temperature. PFA was removed by washing twice in TBS (10mM Tris, 100mM NaCl pH 7.5), then permeabilised by incubation with TBS containing 0.1% NP-40, for 4 min, detergent was then promptly removed by washing twice with TBS. Non specific binding was blocked by incubation with TBS containing 10% pooled human AB, 15 min, room temperature, after which the primary antibody was applied in TBS plus 1% human AB for 1hr, gently rocking. Non-bound primary antibody was removed by washing 3 times with TBS plus 1% human AB serum, 5 min rocking, before application of Alexa™ dye-conjugated secondary antibody, in TBS plus 1% human AB, 1hr, rocking. Cells were again washed x3 in TBS plus 1% human AB, before mounting in either 50% glycerol, or AntiFade™ reagent (Molecular Probes, Oregon, USA), protocol as manufacturers instructions. Slides were then examined by fluorescence microscopy, and images captured using AppleMac OpenLab image capture system.

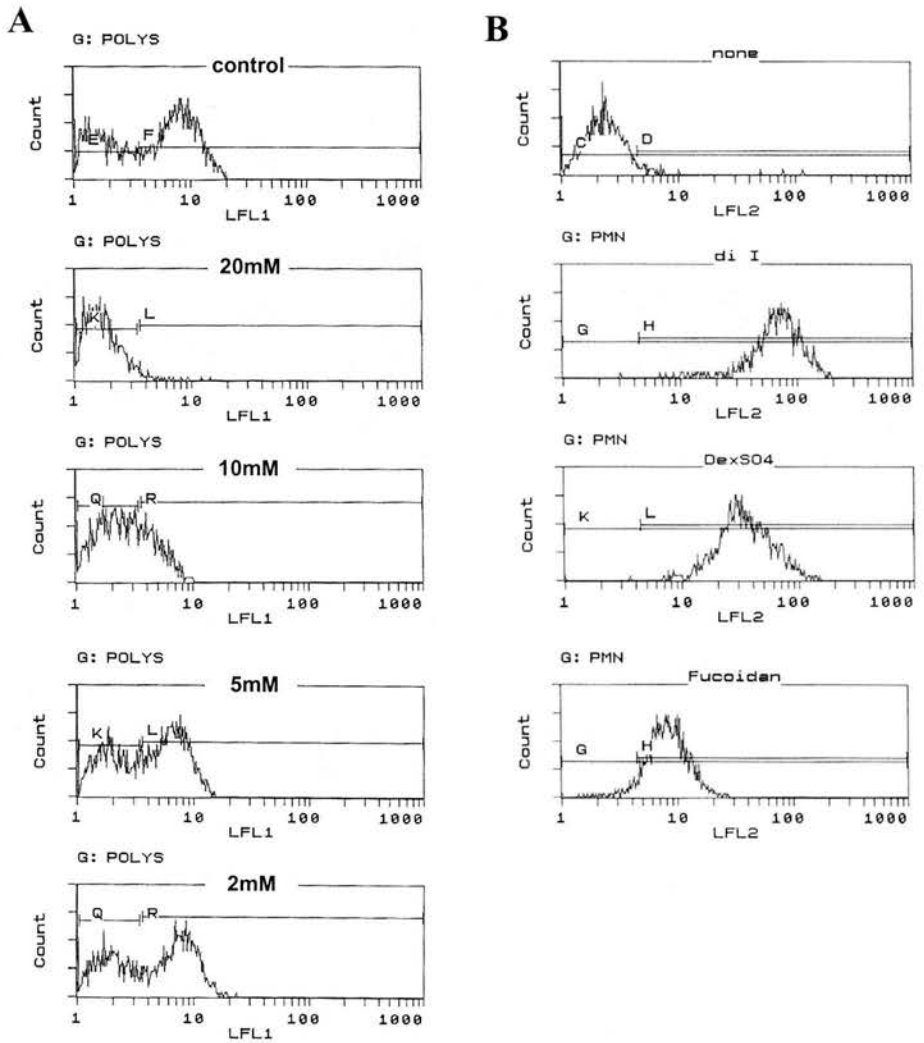


Figure 4. Validation of inhibitors

To demonstrate the functionality of inhibitors of phosphatidylserine receptor and scavenger receptors we assessed the inhibition of FITC-conjugated Annexin V binding to apoptotic neutrophils with phospho-L-serine, and inhibition of macrophage uptake of dil labelled-acetylated LDL with dextran sulfate and fucoidan by flow cytometry. **A** Profile of Annexin V binding (at sub-saturating concentration, 1:2500) to apoptotic neutrophils in the presence of 2-20mM phospho-L-serine. **B** Macrophage uptake of dil-acLDL (1μg/ml) in the presence of 200μg/ml dextran sulfate, and 500μg/ml fucoidan.

Immunoprecipitation and western blotting

Monocyte-derived macrophages were cultured for 5 days ± 1μM DX in 6-well tissue culture plates as described above. Cells were washed with TBS containing 0.1mM $NaVO_3$ plus protease inhibitor cocktail (Boehringer Mannheim, Germany), and lysed by incubation with 150μl/well of lysis buffer (TBS:10mM Tris, 100mM NaCl pH 7.5; 1% NP-40 (v/v), 0.1mM $NaVO_3$, and protease inhibitor cocktail), or 120μl RIPA buffer (for composition see *Rac assay*), 10 minutes on ice. Membrane and nuclear material was removed by centrifugation at 14,000 x *g*, 4°C, for 30 minutes, and supernatants were "pre-cleared" of macrophage Fc receptors by incubation with protein-A agarose-coupled rabbit anti-mouse IgG, 4°C, 30 min, followed by centrifugation at 15,000 x *g*, 3 min, 4°C (immunoprecipitations only). 10μl of lysate was removed and protein concentration estimated using a detergent compatible protein estimation kit (Pierce, Illinois, USA) (protocol as manufacturers instructions). Lysates were equilibrated to contain equivalent levels of protein by dilution with additional lysis buffer, and 100μl of lysate (100-150μg total protein) was incubated with 1μg of either mouse IgG control, anti-paxillin or pyk2 mAb, at 4°C, for 30 minutes, shaking. Immune complexes were precipitated by incubation with 15μl of protein-A coupled rabbit anti-mouse IgG (Sigma), 30 min, 4°C, shaking. Immunoprecipitates were washed twice in TBS pH7.5 plus 0.1% Triton X-100, for removal of proteins non-specifically bound, and once in 25mM Tris pH 8.0 plus 0.05% SDS, to remove salt. Samples were then boiled for 5 min in 15μl of 2x reducing buffer (0.5M Tris pH6.8, 4% w/v SDS, 30% v/v glycerol, 0.1% w/v bromophenol blue, 1% β-mercapthoethanol), resolved on a 9% polyacrylamide gel (37.5mM Tris pH8.8, 0.1% w/v SDS, 0.2% w/v TEMED, 0.04% w/v APS) under reducing conditions (0.1 % β-mercaptoethanol), and transferred electrophoretically (50V for 1 hour) onto nitrocellulose (Amersham Pharmacia Biotech) in 25mM Tris, 192mM glycine pH8.3, 20% w/v methanol. For detection of phosphotyrosine, membranes were blocked with TBS plus 0.05% Tween-20 (TBS-T) all other blots with TBS-T plus 10% non-fat dried milk powder (w/v).
For antibody concentrations see individual figure legends.

Assay for detection of activated Rac

Adherent macrophage cultures were lysed in RIPA buffer (50mM Tris pH 7.2, 500mM NaCl, 10mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Boehringer Mannheim, Germany) plus 1mM PMSF. Lysates were cleared of membrane and nuclear material by centrifugation, total protein was estimated and levels equilibrated as described for immunoprecipitation. 20µl of lysate was removed for estimation of total Rac protein and the remaining protein (approximately300µg) was incubated with GST-PAK (CRIB) fusion protein coupled to Sepharose beads, 4°C, 1hr, shaking. Beads were washed 4 times in ice-cold Tris buffer (50mM Tris pH7.2, 150mM NaCl, 10mM $MgCl_2$, 1% Triton X-100, protease inhibitor cocktail, 1mM PMSF), and the amount of active Rac bound to PAK CRIB domain quantified by SDS PAGE and western blotting as described for immunoprecipitation.

RNA isolation and RT-PCR

Macrophages were washed once in ice-cold PBS, lysed and RNA extracted using Trizol (Gibco BRL, Paisley, UK) (protocol as manufacturer's instructions). RNA was DNAse treated to remove genomic DNA for 1h, 37°C, and the resulting RNA used in RT-PCR reactions using Gibco One step™ RT-PCR kit (protocol as manufacturers instructions). Reactions proceeded for 35 cycles, with annealing at 53°C for M-DOCK, DOCK180 and hABC1, 54°C for hCed6; and 53/54°C for Actin.

DOCK180	Forward 5'GAGGCAGAGGAGACGAACAG
	Reverse 3'AAGCCGATTCGGTGTAGTTA
M-DOCK	Forward 5'TGCTGAAGTGGCGTATGAAG
	Reverse 5'CCTCGACCGAACAATGAACT
hABC1	Forward 5'AGCTGCTGGATGAGAGGAAG
	Reverse 5'GGATAGGGCATCTGTTGCAT
hCed6	Forward 5'CCTCATCATGAACCGTGCTT
	Reverse 5'GAAGCTGGCAATTGTGTTGA
Actin	Forward 5'CCACCAACTGGGACGACATG

Reverse 3'GTCTCAAACATGATCTGGGTCATC

Molecular Biology

Amplification of p130cas

A 2.7kb transcript corresponding to the coding region of p130cas was amplified from pUNI-V5-His-p130cas plasmid using *Proofsprinter*^{TM} DNA polymerase (Hybaid-AGS, UK), protocol as manufacturers instructions. In brief, reaction mix (50µl total) contained 5µl 10x buffer, 0.1% Tween-20, 2% DMSO, 400nM 5' primer, 400nM 3' primer, 10ng template, dNTP-Mix (200µM each nucleotide, final concentration), 2.5 units *Taq/Pwo* Mix, ddH_2O as required. The following cycling conditions were used: 1 cycle 2min/94°C; 30 cycles 30sec/94°C, 30sec/60°C, 3.5min/72°C; 1 cycle 10min/72°C. Primers:

Forward 5'AAACCGGGTACCATGAACCACCTGAACGT

Reverse 5'GGCTTACCGAGCTCGAGAATTGCCCT

Purification of PCR products

PCR fragments were purified either directly from the reaction mixture or run on a TAE (Tris-acetic acid-EDTA) buffered 1% agarose gel to separate bands, the specific bands excised using a clean scalpel blade, then purified using QIAquick spin columns (Qiagen, Crawley, UK), protocol as manufacturer's instructions.

Restriction Digest

DNA was digested in a total volume of 30-50µl, depending on the amount of material, using 0.5-1µl of restriction enzyme per reaction(Promega, Southampton, UK), in addition to 3-5µl 10x reaction buffer, for 2hr, at 37°C.

A Tailing

Volumes for one reaction, but may be scaled up depending on the concentration of the PCR product. 7µl of PCR fragment, 1µl 10x *Taq* DNA Polymerase buffer containing $MgCl_2$ (final concentration 2.5mM), 1µl dATP (final concentration 0.2mM), 1µl (5 units) *Taq* DNA polymerase, were incubated for 30 min at 70°C. Tailed PCR product was then purified using a PCR spin column to remove *Taq*, and dNTPs that may inhibit consequent ligation reactions.

Ligation Reaction

Plasmid-insert ligations were carried out in a total volume of 10µl, in 0.5 ml eppendorf tubes to minimise adsorbance of DNA to the tube. "Sticky-ended" reactions were incubated as a 3:1 insert:vector ratio, "blunt-ended" ligations as a 4:1 ratio, calculated using the equation below, in a reaction mix containing 1 µl 10x ligation buffer, 1µl (5 units) of DNA ligase, overnight at 4°C.

(ng of vector x kb size of insert)/ (kb size of vector) x (insert:vector ratio) = ng of insert

Preparation of competent cells

A single colony of the *E.coli strain,* TOP10 was picked and incubated O/N in 10 ml of antibiotic free LB media (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl, pH 7.0), at 37°C, 220rpm. 100ml of LB medium was inoculated with 1ml of the overnight culture, and shaken at 37°C until an OD_{600} of between 0.6-0.8 was reached. Cells were pelleted by centrifugation at 5,000 x *g*, 5 min, at 4°C, supernatant discarded, and the pellet resuspended on ice, in ~50ml of ice cold 100mM $CaCl_2$. Cells were pelleted again at 4°C, then resuspended in 4mls of 100mM $CaCl_2$, on ice. Competent cells retained activity for approximately 10 days when stored at 4°C.

Bacterial Transformation

Transformation of BL-21s (Stratagene, La Jolla, USA). A 50µl aliquot of cells was incubated with β-mercaptoethanol (final conc 25mM), for 10 min on ice, with gentle mixing every two minutes. 1µl of plasmid DNA was then added, and cells incubated for a further 30mins on ice. Cells were then heat-shocked by incubation at 42°C for 50 sec, followed by incubation on ice for a further 2 min. 450µl of SOC medium (Stock: 2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.05% w/v NaCl, 2.5mM KCl, immediately prior to use add $MgCl_2$ (10mM final conc) and glucose (20mM final conc)) was then added to the transformation, and cells shaken at 220rpm, 37°C for 1.5 hours. Cells were then pelleted and resuspended in 200µl SOC, and100µl of the culture was plated on LB agar containing 100µg/ml ampicillin, and incubated O/N at 37°C. BL-21 have a poor transformation efficiency, therefore TOP10 *E.coli* strain was used for cloning. Transformation of TOP10 was carried out as described above except β-mercaptoethanol was not included, 5-10µl of ligation reaction was incubated with cells, and TOP10s heat shocked for 60-90 sec. To enable blue/white selection for vector insert during cloning of PCR products, ampicillin plates were spread with 20µl of IPTG (100µg/ml), plates incubated for 30 min at 37°C to allow absorption, then 35µl of X-gal (50mg/ml), and plates incubated for 30mins prior to plating of bacteria.

Mini prep of plasmid DNA

Single colonies were picked and incubated in 2mls LB broth containing100µg/ml ampicillin, O/N, 37°C, 220rpm. Cells from 2mls of culture were pelleted by centrifugation at 5,000 x *g*, 5 min, in 2ml eppendorfs, and plasmid DNA extracted using Promega Wizard™ mini-prep kit (protocol as manufacturers instructions).

Midi prep of plasmid DNA

A single bacterial colony from freshly streaked plates was picked and a starter culture grown in 3mls of LB broth plus antibiotics, for ~8 hrs or O/N in 10mls of LB

plus antibiotics, 37°C, 220rpm. Approximately 100µl of the starter culture was then used to inoculate 25mls of LB broth containing ampicillin, cells were grown O/N, 37°C, 220 rpm. Bacteria were harvested by centrifugation at 5,000 x *g* for 10 min at 4°C, and DNA extracted using Qiagen Plasmid Midi purification kit (protocol as manufacturers instructions). In brief, cells were resuspended in a Tris-EDTA buffer containing RNase A, and lysed by the addition of NaOH and SDS. Genomic DNA, protein, other cell debris and SDS was removed by precipitation with potassium acetate pH5.5, and centrifugation at 10,000 x *g*, 15min. The resulting supernatant was applied to a column pre-equilibrated with a low salt buffer at neutral pH, which allowed binding of plasmid DNA to anion exchange resin. Plasmid DNA was then eluted with a high salt buffer, and isopropanol precipitated.

Primer Design

Primers were designed either with Primer3 program http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi or by eye using the following considerations. Primers were ~20 nucleotides in length for sequencing, and RT-PCR, and ~25 nucleotides for insertion of cloning sites, with a GC content of 40-50%, and melting temperature (T_m) of around 60°C, calculated by the formula: T_m = 69.3 + (0.41 x GC%) - (650 / length). Primers were checked for homology to other sequences by BLAST analysis of nucleotide sequence database http://www.ncbi.nlm.nih.gov/BLAST/, and scanned by eye to ensure no intra or inter-primer annealing would occur.

Sequencing: p130cas construct was sequenced using an ABI sequencer by Nina Kotelevtseva, using the following primers:

1) 5'GTCGGGATCTGTACGACGATG (forward)
2) 5'AGCAGGACGAGTACGACACC (forward)
3) 5'GGCACATCGTAGGTCTCCTC (reverse)
4) 5'GTGAACGGGTGCTTCCTC (forward)
5) 5'CATGTGCCACCAGCGTCT (reverse)
6) 5'GGTCAGTGTGGTCAGGTTGG (reverse)
7) 5'CCAACCTGACCACACTGACC (forward)

8) 5'AAGCTTCGAATTCACCGCATG (reverse)

Production of Fusion Proteins

Isolation of high expressing clones

pTAT-HA-p130cas and pTAT-HA-βgal were transformed into *E.coli* strain BL21 as described above, and plated onto LB agar containing 100μg/ml ampicillin. Six colonies from each transformation was selected and grown in 2 ml LB broth plus 100μg/ml ampicillin, O/N. The following day 5mls of LB broth plus ampicillin was inoculated with 500μl of O/N cultures, and incubated at 37°C, 220 rpm until OD_{600}=0.5. Protein expression was induced by the addition of IPTG to a final concentration of 1mM (stock 1μM in dimethylformamide), followed by incubation at 37°C, 220 rpm, for 5hrs. Cells were harvested by centrifugation at 4,000 x *g*, 20min, 4°C. Cell pellets were resuspended in 400μl of lysis buffer (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 8.0) by vortexing at room temperature, until solutions were translucent, and clarified by centrifugation at 10,000 x *g*, 30 min. Supernatants were then incubated with 10μl of Ni-NTA slurry (Qiagen, Crawley, UK), pre-equilibrated with lysis buffer, shaking for 1hr at room temperature. Ni-NTA beads were pelleted at 15,000 x *g* for 3 min, and washed twice with buffer C (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 6.3), followed by resuspension in SDS-PAGE loading buffer. Samples (plus aliquots of whole cell lysates prior to purification and buffer C wash for comparison) were then analysed by SDS-PAGE and either western blotting with mAb specific for p130cas and the HA epitope, or direct staining of acrylamide gels with Coomassie-brilliant blue dye.

Bulk production of TAT-fusion proteins: His-Tag purification

A single colony from freshly streaked plates was picked and grown O/N in 10 ml LB broth plus ampicillin for both p130cas and β-gal fusion proteins. 500ml and 1L of LB-broth containing ampicillin was inoculated with 5ml and 10 ml of β-gal and p130cas starter cultures respectively. Cultures were incubated at 37°C, 220 rpm,

until an OD_{600} of 0.5 was obtained. Cultures were then induced to express protein with IPTG (final concentration 1mM) for 5 hours, after which cells were harvested by centrifugation at 4,000 x *g*, 20min, 4°C, supernatants removed, and pellets stored O/N at -20°C. Pellets were thawed on ice, resuspended in lysis buffer plus 10mM imidazole (to minimise non specific binding), at 5ml per gram wet weight for β-gal expressing cultures, and 2.5ml per gram for p130cas cultures, as this fusion protein did not appear to be expressed as efficiently as β-gal. To ensure complete lysis, cells were stirred for 60 min at room temperature, or until the solution became translucent, before clarification of lysates by centrifugation at 10,000 x *g* for 30 min. Cleared lysates were then incubated with 1ml of the Ni-NTA slurry (pre-washed with lysis buffer) per 4ml of lysate for 1hr, room temperature, shaking. After fusion protein binding, the resin/lysate mix was decanted into a disposable syringe barrel pre-packed with 0.5-1 cm of nylon wool. Lysate was allowed to pass through by gravity flow, such that the Ni-NTA beads sedimented with fluid flow, creating a resin column. The column was washed with lysis buffer until the OD_{280} of the elutant was <0.01, followed by buffer C, until OD_{280}= <0.01. Fusion protein was then eluted 4 times with 2ml of buffer D (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 5.9) which elutes monomeric proteins, followed by 4 x 2ml elutions with buffer E (100mM NaH_2PO_4, 10mM Tris, 8M urea, pH 4.5) which elutes multimers. Samples from each step were analysed by SDS-PAGE together with western blotting, or Coomassie-blue staining of acrylamide gels.

Purification of fusion proteins by ion-exchange

Pooled fractions of fusion proteins from the Ni-NTA column were diluted with HEPES buffer and NaCl to a final concentration of 4M urea, and 50mM NaCl. Samples were applied to a 10ml Q-sepharose column pre-equilibrated with 40ml of low salt buffer (50mM NaCl, 20mM HEPES, pH 8.0), and allowed to pass through by gravity flow. The column was washed once with 40ml low salt buffer, and protein eluted into 1ml fractions with 20ml high salt buffer (1M NaCl, 20mM HEPES), in twenty 1ml fractions. 2 µl of each fraction was spotted onto nitro-cellulose membrane and probed with p130cas and HA mAb to determine which fractions contained protein.

Desalting fusion protein samples

Pooled fractions from the Q-Sepharose column were concentrated using Centricon® centrifugation filter columns (Millipore, Massachusetts) (protocol as manufacturer's instructions), to a volume of 2.5ml. Protein samples were then applied to a disposable PD-10 desalting column (Amersham Pharmacia Biotech. (Buckingham, UK)), pre equilibrated with PBS, eluted in PBS and flash frozen in 15% glycerol. Proteins were stored at -80°C before application to cells

Protein Transduction into macrophages

Peripheral blood monocyte derived macrophages were matured by adherent culture for five days ± 1μM dexamethasone in 48 well culture dishes, as described in *cell culture*. Macrophages were washed once in IDMEM before incubation with 100μl/well of pure fusion protein for 15-60 min at 37°C, 5% CO_2. For assessment of protein transduction, supernatants were removed and retained, macrophages washed once in IDMEM, lysed with RIPA buffer and protein transduction assessed by SDS-PAGE and western blotting with mAb specific for p130cas and HA as described previously. Alternatively to assess the effect of fusion proteins on macrophage phagocytic capability, after removal of protein supernatants, macrophages were incubated directly with apoptotic neutrophils without washing to limit cell loss in DX-treated cultures. Phagocytosis was then assessed using the flow cytometric assay as described above.

Statistical analysis

Data were analysed using the GraphPad Instat statistical analysis package. Tests were chosen on the assumption that data were paired, as control and treatment groups were obtained from the same donor, and were from a non-Gaussian distibution. Figure 2, chapter 3 demonstrates the variability of phagocytosis between individual

donors, which we did not regard as being from a normal distribution. For comparison of two data sets a Mann-Whitney test was used and for comparison of multiple data sets a Freidman Test with post test (a repeated measures ANOVA) was chosen. Data describing the cell surface phenotype of DX-treated macrophages was normalised and presented as a percentage of control. For analysis of this data we used a students T-test for analysis, as the data was paired and we assumed were from a population with normal distribution.

CHAPTER 3: CHARACTERISATION OF THE EFFECT OF GLUCOCORTICOIDS ON MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS

Introduction

The normal resolution of inflammation requires both the induction of apoptosis of extravasated cells and the swift removal of cellular corpses by macrophages or other resident phagocytes in a non-phlogistic manner (Akbar *et al.*, 1994; Fadok *et al.*, 1998a; Meagher *et al.*, 1992; Newman *et al.*, 1982; Savill *et al.*, 1989). However The pathogenesis of a number of diseases such as asthma, emphysema, and rheumatoid arthritis are characterised by the persistence of inflammatory cells (Haslett *et al.*, 1994; Weiss, 1989). Dysregulation in either apoptosis or phagocytic clearance may result in the accumulation of inflammatory cells, or secondary necrosis of non-ingested apoptotic cells, potentiating the initial inflammatory insult. Modulation of cell apoptosis programs or macrophage phagocytic capacity for clearance would therefore be of great therapeutic benefit. Cytokines such as TNF, GM-CSF, TGF-β or IL-1 have been demonstrated to augment phagocytosis of apoptotic cells (Ren and Savill, 1995). However the effects of these cytokines on uptake were small and the pleiotropic consequences of some of these agents may restrict their potential therapeutic value. Glucocorticoids are often used for the treatment of many inflammatory conditions due to their capacity for inhibition of inflammatory cell recruitment, and down-regulation of the production and responsiveness of cells to pro-inflammatory cytokines (Schleimer, 1993). In addition they have been shown to differentially modulate granulocyte cell fate (Cox *et al.*, 1995; Meagher *et al.*, 1992). Glucocorticoids reduce the number of circulating eosinophils in patients with hypereosinophilia, and induce eosinophil apoptosis in vitro (Meagher *et al.*, 1992). In contrast dexamethasone prolongs neutrophil survival in vivo and in vitro (Cox *et al.*, 1995; Meagher *et al.*, 1992). The induction of eosinophil cell death would account for the profound resolving properties of glucocorticoids, however we would predict that inhibition of neutrophil cell death would not promote resolution, but may

exacerbate an inflammatory response. Death induction must be accompanied by swift clearance by phagocytes, a large induction of apoptotic particles may overwhelm a tissue's phagocytic capacity as seen in mice treated with anti-Fas mAb (Ogasawara *et al.*, 1993). Recent research in *C.elegans* suggests that engulfment promotes cell death (Hoeppner et al., 2001; Reddien et al., 2001), and uptake of apoptotic neutrophils by human macrophages can induce cell death in bystander leukocytes (Brown and Savill, 1999). A delay in neutrophil phagocytosis may allow further recruitment of peripheral blood monocyte/macrophages that can then facilitate the triggering of final death pathways, resulting in efficient removal of extravasated granulocytes before induction of secondary necrosis. Glucocorticoids can alter gene transcription, either by transactivation at glucocorticoid response elements, or transrepression of other transcription factors such as AP-1, and therefore have the capacity to affect expression and function of potential phagocytic uptake mechanisms. A number of cell surface molecules have been proposed to mediate the uptake of apoptotic cells (for review see Giles *et al.*, 2000), these include lectins (Duvall *et al.*, 1985), αvβ3 integrin/CD36/thrombospondin complex (Savill *et al.*, 1990; Savill *et al.*, 1992), phosphatidylserine receptors (Fadok *et al.*, 2000), scavenger receptors (Platt *et al.*, 1996), receptors for oxidised lipids (Chang *et al.*, 1999), CD14 (Devitt *et al.*, 1998), CD29 (Schwartz *et al.*, 1999), the ABC1 transporter (Luciani and Chimini., 1996) and receptors for complement components C3bi (CR3/CR4) (Mevorach *et al.*, 1998), and C1q (Botto *et al.*, 1998). Lui *et al.* (1999) demonstrated that treatment of human peripheral blood monocyte derived macrophages, mouse bone marrow-derived, or thioglycolate-elicited peritoneal macrophages with glucocorticoids for 24 hours significantly augmented the uptake of apoptotic cells, an effect which was mediated via the glucocorticoid receptor, and was protein synthesis dependent. More strikingly, their preliminary evidence showed that the increase in phagocytosis could be further potentiated by treatment of peripheral blood monocyte derived macrophages for a prolonged period, up to 4 days. In this chapter we describe the further characterisation of the effects in phagocyte function elicited by long term glucocorticoid treatment of

monocyte derived macrophages using the synthetic glucocorticoid dexamethasone, and assessment of the involvement of previously described phagocytic uptake mechanisms.

Results

Glucocorticoids augment phagocytosis of apoptotic neutrophils in a time and dose dependent manner

Peripheral blood monocyte-derived macrophages were matured by adherent culture in the presence of 1μM dexamethasone (DX) for varying periods up to 5 days. Phagocytosis of apoptotic neutrophils by the resulting macrophage population was assessed (see Materials and Methods for macrophage characterisation). Preliminary experiments showed that monocyte/macrophages treated for greater than 24 hr detached from culture plastic during the phagocytosis assay preventing accurate assessment of phagocytosis. Careful observation revealed that the detachment appeared to be due to the highly phagocytic nature of glucocorticoid-treated cells. We therefore reduced the duration of the assay from the previously characterised 30 minute (Liu *et al.*, 1999), to a 20 minute assay (Giles *et al.*, 2001). In agreement with the observations of Lui *et al.* phagocytosis was found to increase with duration of steroid treatment (fig. 1a), with maximal effect after early inclusion of steroid into the culture medium (untreated: 9.9±0.8%; DX 5d: 39.2±2.7% (mean percent of cells phagocytosing ±SEM, n=68). To further characterise the effect of early glucocorticoid exposure on macrophage phagocytic capacity, peripheral blood monocyte derived macrophages were incubated with 1μM DX for 24 hr periods, beginning at time zero of initial culture, followed by removal of steroid and incubation with media only for the remaining duration of maturation (fig. 1b). Exposure to DX during the first 24 h produced a significant increase in phagocytosis of apoptotic cells, similar to that observed in cultures incubated with DX for the full 5 days of maturation. Interestingly, the induction in phagocytic capacity observed with DX treatment from 0-24 hrs was not always as consistent as DX exposure for 5-days. This observation suggests that although glucocorticoid treatment of monocytes was able to "program" subsequent macrophage phagocytic capacity, the

presence of serum factors also influence macrophage programming in the absence of glucocorticoids. Analysis of the phagocytic index (a function of the number of cells phagocytosing within the population, and the number of particles ingested) for cells incubated with DX for 5 days, demonstrated a seven-fold increase (untreated: 14.1±9.3 (SEM), DX: 99.3±14.9 (SEM) n=11) (fig. 1c). We therefore focused our investigation on five-day DX-treated monocyte/macrophages populations.

Five-day DX treatment consistently augmented macrophage phagocytic capacity above that of control in each experiment. However, the absolute levels of phagocytosis proved to be highly variable between experiments and donors (fig 2). Between 1.7 and 37.7% of cells in untreated populations scored for phagocytosis, whereas in DX treated cultures these values ranged from 7.4 to 85.6%. Despite such different levels of phagocytosis, the relative response to varying concentrations of DX was correlative (fig 3), with maximal phagocytosis reached after ~500nM. Previous observations indicated that the percentage of apoptotic cells in the phagocytic "meal" (typically 50-80% positive by AnnexinV staining) did not significantly alter the phagocytic potential of the macrophage population (S.Hart, personal communication). Furthermore, comparisons between uptake of apoptotic cells which were syngeneic or allogeneic to the macrophage population did not appear to account for this variation. For example donor KG recorded 10.2% (control) and 25.7% (DX) phagocytosis of allogeneic targets compared to 8.9% (control) and 29.1% (DX) uptake of syngeneic cells.

DX augmentation of phagocytosis of apoptotic neutrophils acts via the glucocorticoid receptor

In order to investigate if 5-day DX augmented phagocytosis was acting via the classical glucocorticoid receptor pathway we incubated peripheral blood monocytes with 1μM DX for five days (as previously described) with the addition of 10μM RU38486, a synthetic antagonist of glucocorticoid receptor signalling. RU38486 inhibited DX augmented phagocytosis to levels comparable with untreated cultures (fig. 4) (DX:

42.9±5.8%; DX+RU38486: 8.1±0.5%, untreated 7.6±1.1 (mean ±SEM n=5)), confirming that the effect of DX was mediated via the glucocorticoid receptor. Interestingly, RU38486 attenuated baseline phagocytosis in non-DX treated cultures from 7.6±1.1% to 5.2±1.2% (mean ±SEM). This trend suggested an influence of endogenous glucocorticoids from autologous serum in the culture medium, possibly contributing to the variation in phagocytosis observed between donors when monocytes are cultured in autologous serum alone. As the 5-day DX effect was mediated through the glucocorticoid receptor it was probable that changes in protein expression elicited the phagocytic phenotype. We were unable to test this hypothesis by the addition of inhibitors of translation during the culture period as this prevented normal monocyte to macrophage differentiation (data not shown). However, the use of synthetic steroids with predominately transactivating or transrepressing activity allowed us to investigate this possibility further.

Dexamethasone augmented phagocytosis of apoptotic cells requires both transactivation and transrepression activity of the glucocorticoid receptor

DX induces both transactivation and transrepression of gene transcription. In order to investigate if DX augmented phagocytosis was protein synthesis-dependent we utilised synthetic glucocorticoids which had previously been characterised to have predominately transactivational (ZK57740 and ZK77945) or transrepressional (RU24782 and RU24858) activity (Heck *et al.*, 1997; Vayssiere *et al.*, 1997). Monocyte/macrophages were cultured for five days (as described above) ± synthetic steroids, and the effects on phagocytosis of apoptotic neutrophils assessed. At low concentrations (10-100nM) synthetic steroids did not significantly alter phagocytosis of apoptotic neutrophils (fig. 5). However at 1μM both transrepressors and one transactivator (ZK77945) augmented phagocytosis above control levels, but not to the same extent as DX, with values for ZK77945 and RU24858 showing statistical significance. This suggests both glucocorticoid transactivating and transrepressing activity is required for augmented phagocytosis.

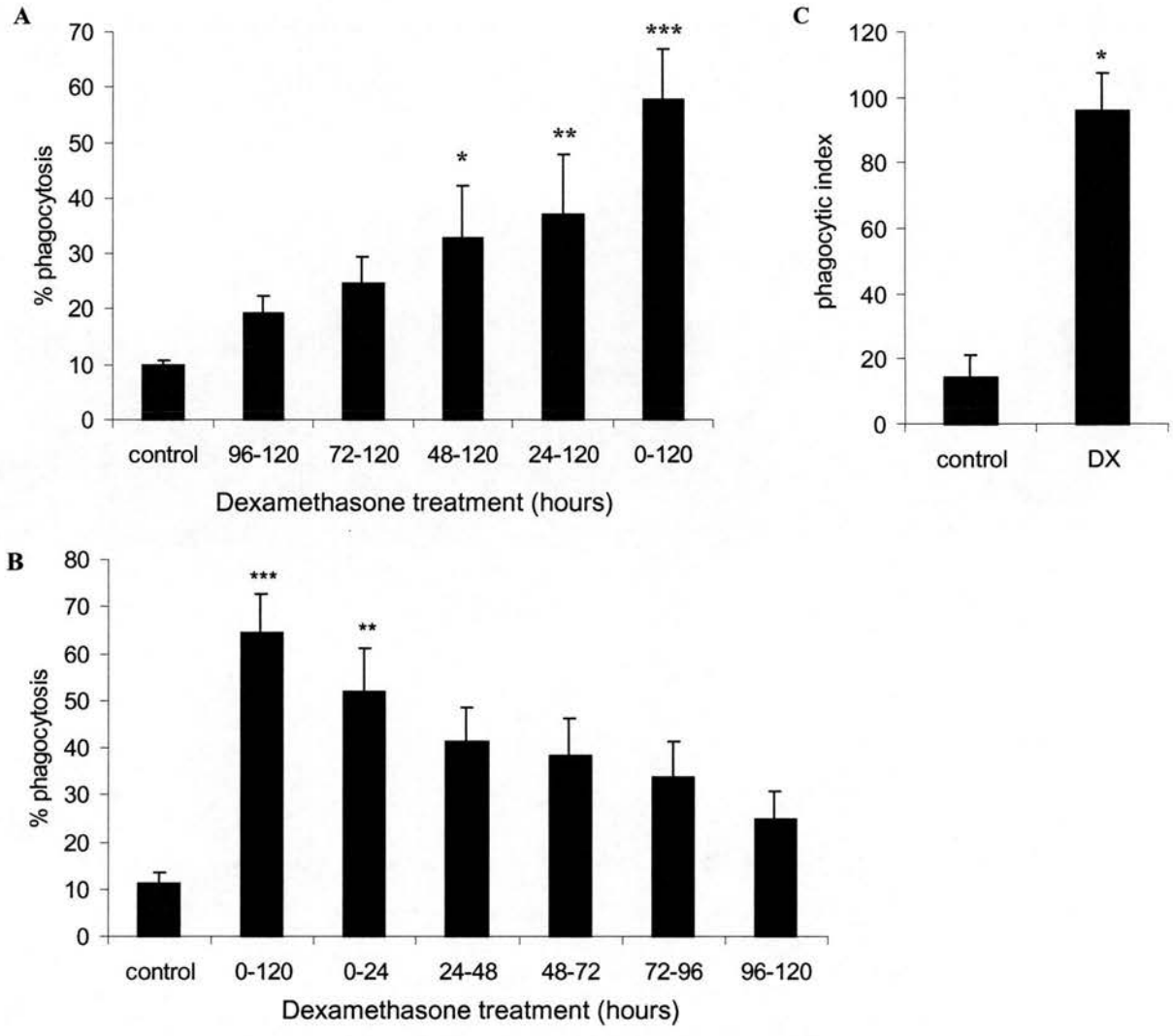


Figure 1. Effects of dexamethasone on macrophage phagocytosis of apoptotic neutrophils.

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM dexamethasone (DX) for varying periods of time. The capacity for phagocytosis of apoptotic neutrophils was determined in a 20 minute assay by microscopic visualisation of myeloperoxidase activity. **A** Monocyte/macrophages were incubated with the addition of 1μM DX for up to 5 days. Data shown as mean phagocytosis ±SEM for 6 separate experiments. **B** Monocyte/macrophages were exposed to a 24hr window of glucocorticoid treatment over a 5-day period. After DX treatment medium was replaced and macrophages cultured in the absence of steroid. Mean phagocytosis ±SEM, $n = 5$. **C** Phagocytic index (calculated as [average number of neutrophils phagocytosed / macrophage] x [% macrophages phagocytosing one or more neutrophils] for untreated and 5 day DX-treated monocyte/macrophages (values taken from mean of a minimum of 500 cells, per well, in duplicate wells ±SEM, $n = 5$). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control values, Friedman Test with post test (A, B); Wilcoxon Matched pairs (C).

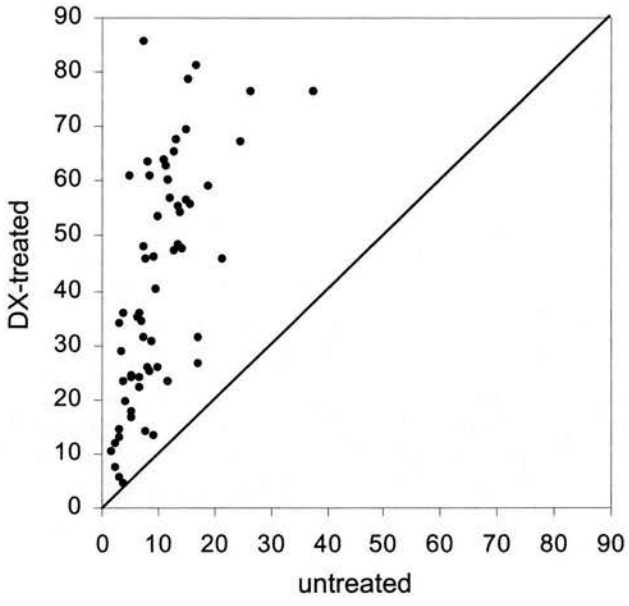


Figure 2. Phagocytosis levels of untreated and DX-treated macrophages are highly variable

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days prior to assessment of phagocytic capacity as described in figure 1. Data distribution of all untreated vs 5-day DX treated phagocytosis assays (n= 68).

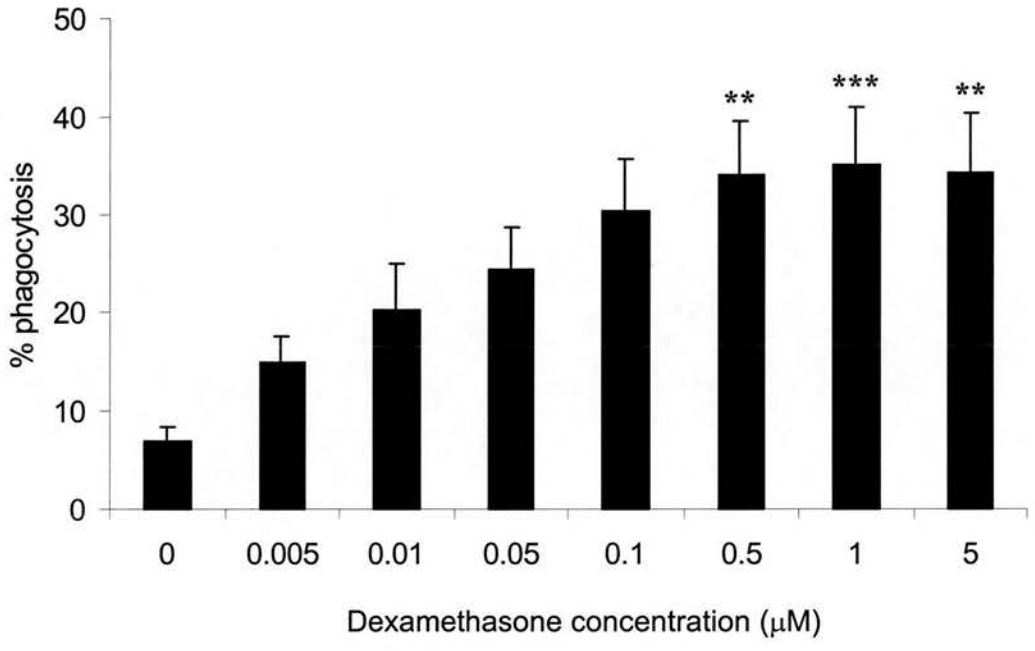


Figure 3. Dexamethasone concentration response
Monocyte-derived macrophages were incubated for 5 days in the presence of different concentrations of DX, and phagocytic capacity for apoptotic neutrophils assessed as for figure 1. Data shown as mean phagocytosis ±SEM, n = 5. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs control values, Friedman Test with post test.

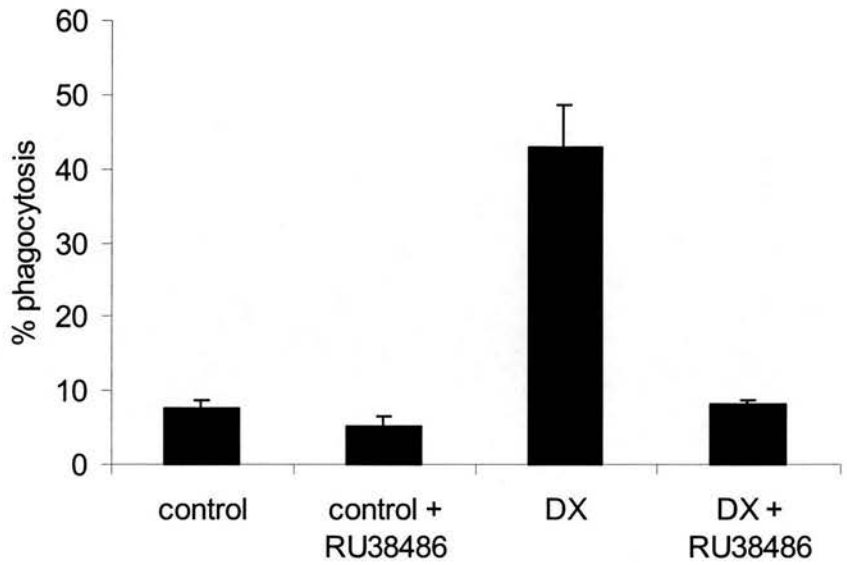


Figure 4. DX functions through the classical glucocorticoid receptor pathway
Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM dexamethasone, and 10μM RU38486 for 5 days, and phagocytosis of apoptotic neutrophils assessed as for figure 1. Data shown as mean phagocytosis ±SEM, n = 5.

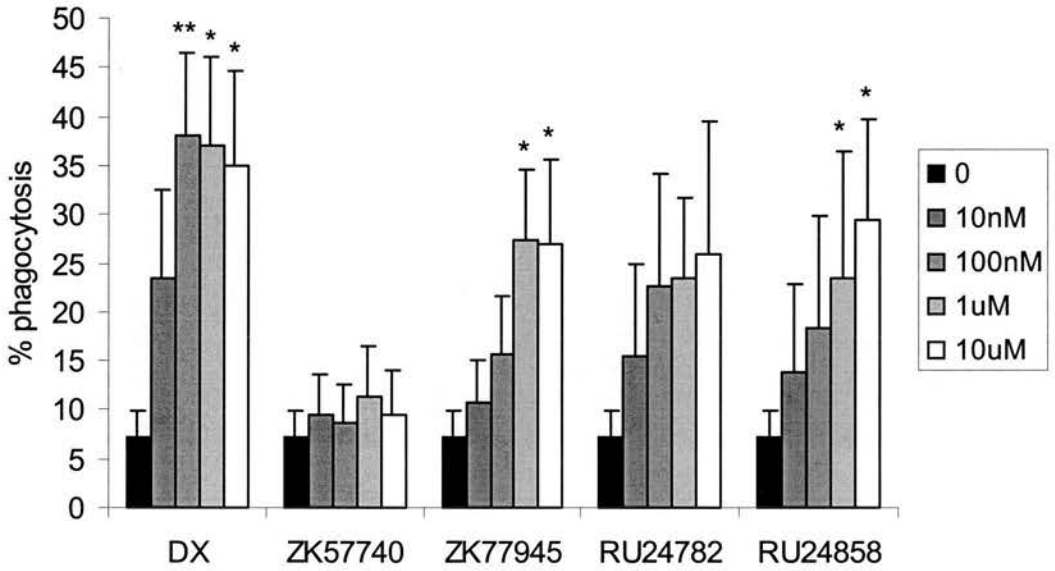


Figure 5. Effect of synthetic glucocorticoid analogues with predominately transactivating or transrepressing activity on macrophage phagocytosis of apoptotic neutrophils.

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of varying concentrations of DX or the transactivating glucocorticoids: ZK57740 and ZK77945, and transrepressing glucocorticoids RU24782 and RU24858, for 5 days, and phagocytosis of apoptotic neutrophils assessed as before. Data shown as mean phagocytosis ±SEM, n = 3-6 (*, $p < 0.05$; **, $p < 0.01$ vs control values, Friedman Test with post test)

Increased phenotypic and morphological homogeneity in dexamethasone treated cultures

A number of macrophage cell surface molecules have been implicated in the recognition of apoptotic cells (see Giles *et al*, 2000 for review, and chapter 1, table 3). To determine whether glucocorticoids augmented phagocytosis via an upregulation of previously described systems, we first assessed the effects of DX upon receptor expression of a panel of molecules through mAb binding and flow cytometry. Monocyte/macrophages were cultured for 5-days in the presence or absence of DX as previously described. Flow cytometric analysis revealed no change in the percentage of cells within the population expressing phagocytic receptors following DX treatment. However, expression profiles for several molecules including CD14 and HLA-DR, appeared more homogenous after treatment with DX (fig. 6). Analysis of the coefficient of variation for the fluorescence peaks of each mAb staining confirmed this impression with decreases in the range of expression of CD14 (108±14; 60±5, n=13), CD16 (96±8; 62±4, n=11) HLA-DR (122±11; 96±6, n=10), CD44 (99±15; 66±7, n=6) and CD51 (81±5; 53±4, n=11) (untreated versus DX-treated ±SEM). Additionally, analysis of forward and side scatter profiles suggested DX-treatment also produced a morphologically more homogenous population of cells (fig. 6). This effect of glucocorticoid treatment on macrophage morphology will be explored in chapter 4.

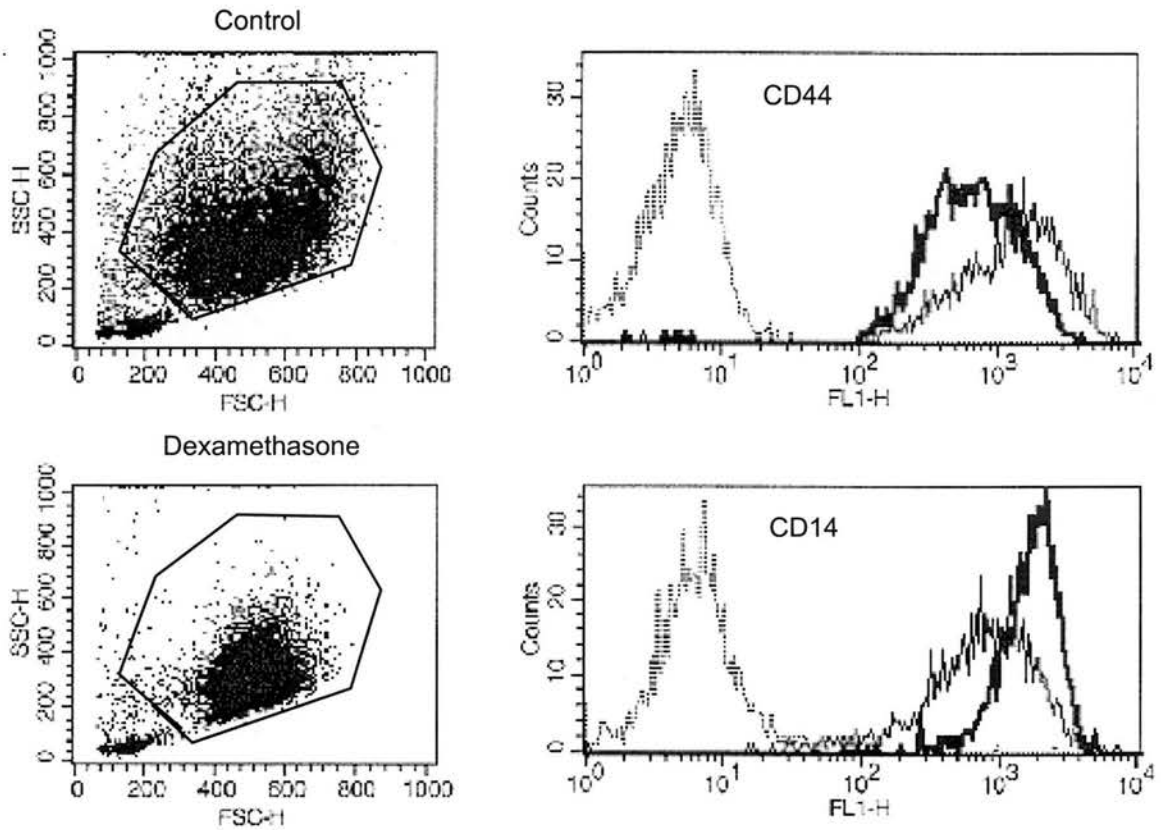


Figure 6. Increased phenotypic and morphological homogeneity of DX-treated monocyte/macrophage cultures

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days. Monocyte/macrophage were removed from culture plastic by incubation with EDTA on ice, and surface phenotype was examined by indirect immunofluorescence using isotype control, CD44 mAb (5A4) or CD14 mAb (UCHM1) together with flow cytometry. The profiles (from one of at least 4 separate determinations) shown here illustrate the more homogeneous laser scatter properties of DX-treated macrophages . The fluorescence histograms (FL1-H) for cells within the gates shown illustrate the more uniform cell surface expression of CD14 and CD44 following DX treatment. Dotted lines show binding of relevant isotype controls, solid lines: mAb binding to untreated macrophages, and bold lines: mAb binding to DX-treated macrophages.

Dexamethasone augmented phagocytosis of apoptotic neutrophils utilises multiple pathways

Comparison of mean fluorescence intensity of mAb binding demonstrated DX-induced alterations in the levels of cell surface expression of several molecules (table 1). Changes were not accountable by increased uniformity of expression, as cell surface markers which exhibited decreases in the coefficient of variation had both augmented and attenuated mean fluorescence after DX administration. HLA-DR and the macrophage marker CD16 were slightly elevated after DX treatment, whereas CD44 expression was decreased. CD163, which has homology to the scavenger receptor family of proteins and previously described as a glucocorticoid-inducible protein, was considerably increased after DX treatment (189.9±43.4% of control). In contrast, adhesion molecules ICAM-1 and β_1 integrin subunit CD29 were significantly attenuated by DX treatment to 51.6±5.4% and 44.4±6.3% of control ±SEM (p value, <0.05 using students t-test). To our surprise considering their well documented role in phagocytic processes, DX treatment reduced expression of the mannose receptor, CD44, CD44v3 and the β_3 integrin subunit CD61 to 38.1±6.8%, 58.8±6.4% and 82.7±7.7% of control ±SEM respectively (p value, <0.05 using students t-test). Furthermore, analysis of expression of CD36 using the monoclonal antibodies IVC7 and smϕ suggested a steroid-induced decrease. The mAb IVC7 consistently demonstrated elevated binding when compared with smϕ in untreated cultures (results not shown), suggesting the epitope recognised by smϕ is not present on all CD36 molecules. After DX treatment, mean fluorescence intensity for smϕ binding was not decreased (86.6±20% of control ±SEM), however binding of the pan CD36 mAb was consistently reduced to 58.1±8.1% of control ±SEM. Expression of CD14, and the α_v integrin subunit CD51 was not affected by steroid treatment. Together these results suggest that DX augmented phagocytic capacity was not due to an increase in expression of putative "phagocytosis receptors". However, as surface expression is not an indicator of receptor function, we next used specific mAb/soluble ligand inhibitors of apoptotic recognition pathways, in the

phagocytosis assay, to determine a role of different receptors in DX-treated macrophage phagocytosis.

Monocyte/macrophages cultured for 5-days in the presence of DX as previously described were pre-incubated with soluble inhibitors for 15 min before addition of apoptotic cells, and assessment of phagocytosis was made in the presence of the inhibitor, except mAb which were removed (table 1). Inhibitors of CD36 (smϕ mAb (1:50 ascites) and αvβ3 (0.5mM RGDS peptide) did not prevent phagocytosis of apoptotic neutrophils by DX-treated macrophages (table 1). Smϕ was used at concentrations shown to be saturating by flow cytometry, and RGDS was demonstrated to be functionally active by inhibition of α5β1-mediated T cell adhesion to fibronectin (results not shown). The lack of inhibition by RGDS in DX treated cultures mirrored that for non-DX treated cells in a 30 min assay (percent phagocytosis untreated control 28.5 ± 4%, RGDS 27.5 ± 4%; mean ±SEM n=7 (I. Dransfield personal communication)), suggesting that this pathway does not play a major role in DX-augmented phagocytosis. In addition, 10mM glucosamine (thought to inhibit a charge sensitive mechanism employing $\alpha_v\beta_3$/CD36/TSP) partially attenuated uptake to 63.8±10% of control DX-treated cells, further suggesting that integrin-mediated recognition is not the dominant pathway utilised.

Inhibition of CD14 ligand binding activity with mAb 61D3 (data not shown) and My4 (table 1) also failed to inhibit DX-treated macrophage phagocytosis of apoptotic neutrophils, despite previous reports demonstrating its involvement in peripheral blood monocyte derived macrophage phagocytosis of apoptotic neutrophils (Devitt *et al.*, 1998; Flora and Gregory, 1994; Moffatt *et al.*, 1999). We observed a small inhibitory effect of 2mM phospho-L-serine upon DX-induced phagocytosis of apoptotic neutrophils. Phospho-L-serine exerted a greater inhibition of binding of FITC-labelled Annexin V to apoptotic neutrophils in flow cytometric analysis, demonstrating the compound was functionally active (see Materials and Methods for validation of

inhibitors). However, phospho-L-serine may not be the most effective inhibitor of phosphatidylserine receptor function. Tait and Smith (1999) demonstrated that monomeric phospho-L-serine could not prevent binding of labelled phosphatidylserine vesicles to the monocytic cell line THP-1. Instead, binding could be out-competed by unlabelled phospholipid vesicles containing multiple PS moieties. At the time these studies were performed no blocking mAb to human PS-receptor was available and a role for PS receptor-mediated phagocytosis after DX treatment cannot be discounted.

Glucocorticoid treatment has been reported to increase mannose receptor expression by macrophages (Piemonti *et al.*, 1999). In contrast, our data indicates a decreased expression of the mannose receptor after macrophage exposure to glucocorticoids (table 1), and we were unable to significantly block uptake by inclusion of mannan in the phagocytosis assay (81.5%±5.8% of control ±SEM). Since the mannose receptor was first identified to be involved in uptake of apoptotic cells in fibroblasts (Hall *et al.*, 1994), it may play a more significant role in clearance by non-professional phagocytes.

Phagocytosis was markedly inhibited in the presence of 500μg/ml fucoidan (31.3±12.4% of control, $p < 0.01$ using students t-test) implicating scavenger receptor involvement. Another scavenger receptor ligand, dextran sulphate, partially inhibited phagocytosis (table 1). Both compounds inhibit the recognition of multiple scavenger receptor ligands including oxidised-low density lipoprotein (oxLDL) and acetylated-LDL. Fucoidan inhibited macrophage uptake of diI-labelled acetylated LDL at 1μg/ml to 11.5% and 13.4% of control (untreated and DX-treated respectively). Dextran sulfate was not as potent an inhibitor suggesting it is either a weaker ligand of scavenger receptors, or alternatively has greater specificity. In the latter case, inhibition by fucoidan may reflect the blockade of multiple pathways, including effects on other carbohydrate recognition molecules (for example selectins) (Rosen and Bertozzi, 1994).

	Expression		Function		Refs
	mAb	% relative to control*	Inhibitor	% phagocytosis relative to control*	
$\alpha_v\beta_3$/CD36/thrombospondin					
α_v	13C2	98.7 ±7.3			
β_3	Im6/13	82.7 ±7.7			
$\alpha_v\beta_3$	23C6	83.6 ±7.0	RGDS (active)	85.7 ±5.7 (n=7)	(1,2)
			RADS (inactive)	101.3 ±8.6 (n=3)	
CD36	ICV7	58.1 ±8.1			(1,2)
	smϕ	86.6 ±20.0	smϕ	76.6 ±7.0 (n= 9)	
charge sensitive	-	-	glucosamine	63.8 ±10.0 (n= 7)	(3)
Pattern Recognition Receptors					
mannose receptor	19.2	38.1±6.8	mannan	81.5 ±5.8 (n= 8)	(4)
CD14/ICAM-3	UCHM1	94.9 ±14.4	My4	123±14.5 (n= 2)	(5)
CD44					
CD44 (all isoforms)	5A4	71.6 ±9.0	-	181.6±48.3 (n= 4)	(6)
$CD44_v3$	3G5	58.4 ±6.4	-	-	
Phosphatidylserine receptor	-	-	phospho-L-serine	80.8 ±6.3 (n=8)	(7)
"scavenger" receptors					
SR-A	-	-	dextran sulphate	67.9 ±8.6 (n=10)	(8)
			fucoidan	31.3±12.4 (n=6)	
CD163	BerMac3	189.9 ±43.3	-	-	
ABC binding cassette transporter (ABC1)	-	-	glyburide	44.5±6.1	(9)
other molecules					
HLA-DR	WR18	123.6± 20.6	n.a.	n.a.	
CD16	3G8	130.5 ±11.5	n.a.	n.a.	
ICAM-1	15.2	51.6±5.4	n.a.	n.a.	
β_1-integrin	P4C10	37.1±3.2	n.a.	n.a.	(10,11)

Table 1. Effect of dexamethasone on the expression and function of macrophage "phagocytic receptors"

Monocytes were treated for 5 days with 1μM dexamethasone and the effects upon surface receptor expression and apoptotic cell recognition pathways using previously defined inhibitors of phagocytosis was assessed.

* Note: In this table, "control" receptor expression represents expression on 5 day DX-treated monocyte-derived macrophages relative to untreated 5 day monocyte-derived macrophages. "Control" phagocytosis represents phagocytosis of apoptotic neutrophils by 5 day DX-treated monocyte-derived macrophages in the absence of inhibitor.

References: 1) Savill et al., 1990; 2) Savill et al., 1992; 3) Savill et al., 1989; 4) Hall et al., 1994; 5) Devitt et al., 1998; 6) Hart et al., 1997; 7) Fadok et al., 2000; 8) Platt et al., 1996; 9) Luciani and Chimini, 1996; 10) Schwartz et al., 1999; 11) Erwig et al., 1999

CD44 crosslinking further augments phagocytosis of apoptotic neutrophils in DX treated macrophages.

Crosslinking of the cell surface glycoprotein CD44 specifically increases phagocytosis of apoptotic neutrophils (Hart *et al.*, 1997). The molecular mechanism of CD44 augmentation remains to be determined, and its potential contribution to elevation of phagocytosis by DX is therefore difficult to assess. However, if macrophage phagocytosis after glucocorticoid treatment were independent of a CD44 pathway, CD44 crosslinking would be expected to produce an additive effect on uptake. Additionally, as DX was found to decrease CD44 expression after 5 days treatment, the additive effect might be expected to be more pronounced with decreased exposure to steroid. To test this possibility, monocyte/macrophages were cultured for 5-days with varying exposure to DX (as described for figure 1). Cells were then incubated with the anti-CD44 mAb, 5A4 for 15 minutes, non-bound antibody removed by washing once, and phagocytic capability assessed. CD44 crosslinking was found to augment phagocytosis in both untreated and DX treated cultures (fig. 7a). These results indicate that the level to which CD44 crosslinking was able to elevate phagocytosis did not correlate with the duration of DX exposure, but to the magnitude of the DX response. In assays where 5-day DX macrophages had relatively low phagocytic capacities (33.2% and 50.5%), CD44 crosslinking was able to further augment phagocytosis to 57.2% and 68.3% respectively. However, in one experiment, although CD44 crosslinking was able to elevate phagocytosis in untreated cells (7.6% to 34.4%) and in macrophages treated with DX for 24 hours prior to the assay (31.3% to 51.5%), phagocytosis by 5-day DX treated cells was not augmented (85.6% to 84.3%) probably representing the "phagocytic limit" of the population/assay.

The ATP binding cassette transporter ABC1 is homologous to the *C.elegans* protein CED-7, and is required on the surface of both the phagocyte and the apoptotic particle for uptake (Hamon *et al.*, 2000). ABC1 is thought to mediate the flip-flop of phosphatidylserine in the membrane of the apoptotic cell, although its role in the phagocyte is as yet uncharacterised, its ability to regulate membrane lipids may be

important. Exposure of PS on the surface of macrophages has been reported to be required for uptake of apoptotic thymocytes(Callahan *et al.*, 2000). 100μM glyburide markedly inhibited the uptake of apoptotic neutrophils in both untreated and DX treated cells (untreated + glyburide 41.5±11.0%; DX + glyburide 44.5±6.1% (of control ±SEM n=4-7)) (fig. 7 and table 1). However to our surprise, glyburide treatment only partially inhibited phagocytosis by untreated cells crosslinked with CD44 to 79.2±5.0% of control (SEM) (fig. 7b), suggesting a fundamentally different mechanism of augmentation between DX and CD44 cross-linking.

One interpretation of the data presented is that is that DX-augmented phagocytosis may involve multiple receptor pathways acting co-operatively, or the engagement of a novel receptor pathway which promotes more efficient apoptotic cell recognition. Alternatively, DX may increase overall phagocytic capacity of the cell by altering the intracellular machinery required for the uptake of apoptotic cells.

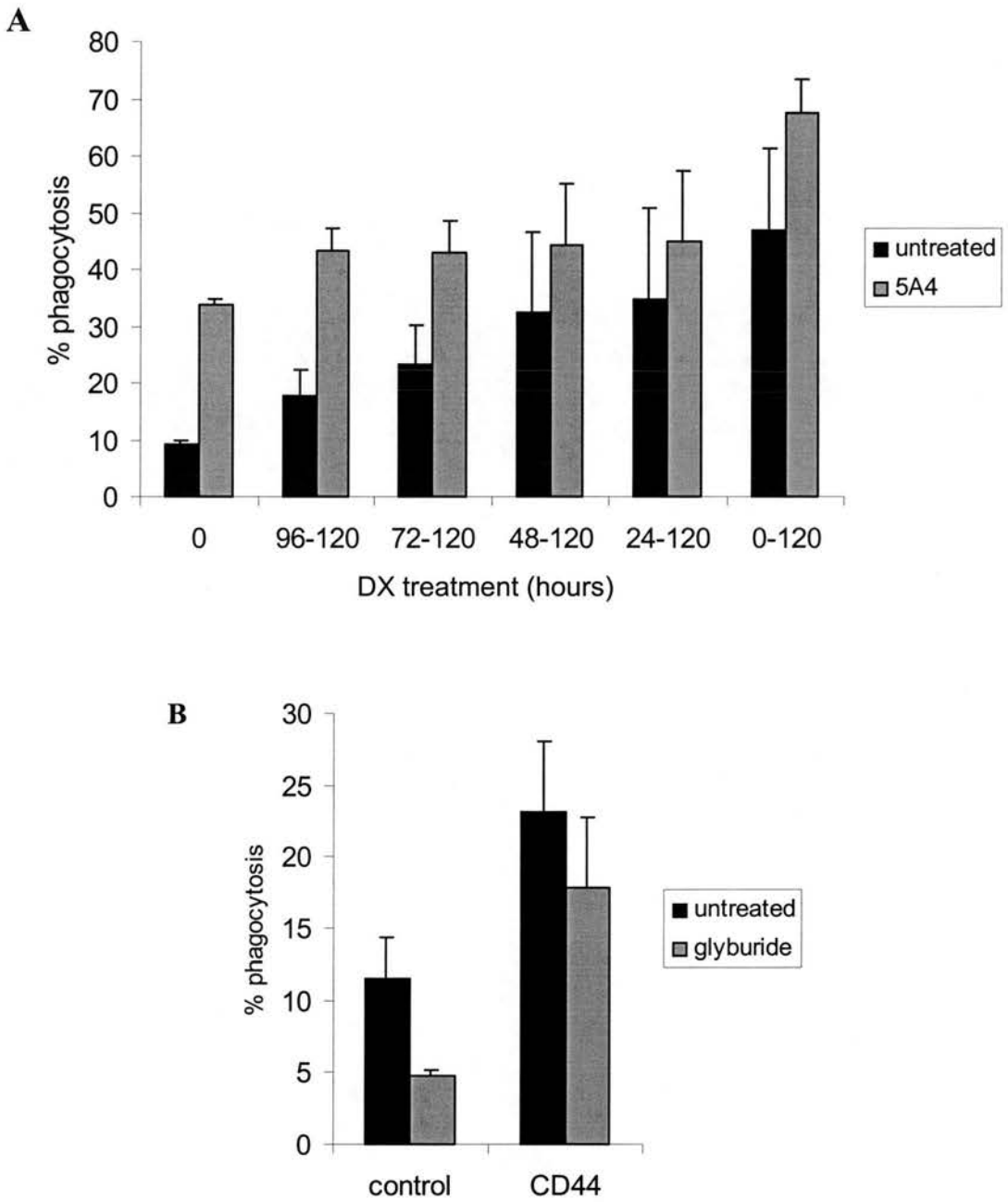


Figure 7. CD44 crosslinking augments phagocytosis of apoptotic neutrophils by DX treated macrophages

A Adherent peripheral blood monocyte-derived macrophages were cultured in the presence of 1μM dexamethasone for the periods shown. Macrophages were incubated with media alone or anti-CD44 mAb 5A4 for 15 min, washed to remove non-bound antibody, and phagocytosis of apoptotic neutrophils assessed as for figure 1. Data shown as mean phagocytosis ±SEM, n= 4. **B** Monocyte derived macrophages were incubated in the absence of DX for 5 days. Macrophages were exposed to 100μM glyburide for 15 min prior to CD44 mAb binding and assessment of phagocytosis as for panel A. Data shown as mean phagocytosis ±SEM, n = 3.

Discussion

Glucocorticoids augment phagocytosis of apoptotic neutrophils

Lui *et al* (1999) described short-term exposure of monocyte-derived macrophages to the glucocorticoid, methylprednisolone (24hours prior to the assay) increased phagocytosis of apoptotic neutrophils. In this chapter I have expanded the preliminary observation that the elevation in phagocytic capacity can be further augmented by an increased exposure to glucocorticoids, and characterised the augmentation of macrophage phagocytosis of apoptotic neutrophils after 5-day DX-treatment. The greatest effect on uptake of apoptotic neutrophils occurred when steroid was added to the culture medium immediately following isolation, suggesting an early event during monocyte to macrophage differentiation promotes the development of a pro-phagocytic macrophage phenotype. This hypothesis will be further explored later in this thesis. Monocyte functional plasticity allows the formation of diverse macrophage phenotypes in response to stimuli encountered in different tissue types or during the course of an inflammatory response. For example, culture in the presence of IL-4 and GM-CSF promotes the formation of an "immature" dendritic cell phenotype (Chapuis *et al.*, 1997; Sallusto and Lanzavecchia, 1994). Autologous serum used for the culture of monocyte/macrophages is likely to contain a number of cytokines including GM-CSF. Assessment of the influence of glucocorticoids on macrophage maturation has not been extensively studied. It is unlikely that the highly phagocytic phenotype induced by DX treatment is a result of an inhibition of monocyte to macrophage differentiation, as macrophages matured in the presence of DX expressed $\alpha_v\beta_3$ and CD16, and in our experience monocytes phagocytose apoptotic cells poorly (data not shown). Previous reports have documented an induction of monocyte cell death by dexamethasone (Schmidt *et al.*, 2001; Schmidt *et al.*, 1999). We found no difference in the number of cells recovered after 5 days between DX-treated and untreated cultures (control: 152±16 cells/field, DX: 140±16 cells/field; (mean ±SEM, n=10). These apparently discrepant results may be accounted for by the higher percentage of autologous serum used in this study favouring monocyte to macrophage differentiation (Musson, 1983), compared to 1% heat inactivated pooled

human AB serum used in the Schmidt study. Together, this data indicates that it is unlikely that selection of cells highly phagocytic but resistant to steroid induced death can account for the phagocytic phenotype.

Levels of phagocytosis are highly variable

Throughout the study we encountered a large variability in both baseline phagocytosis of untreated cultures and DX treated populations. After isolation from peripheral blood, monocytes are selected by adherence to tissue culture plastic for 1 hr, after which the non-adherent lymphocyte population was removed by washing. A residual number of lymphocytes will persist in the culture for 2-3 days. During this period lymphocytes may secrete a number of cytokines including IL-10, IFNγ and IL-4, which can affect both monocyte viability (Estaquier and Ameisen, 1997), and macrophage phagocytic capacity (Erwig *et al.*, 1998; Ren and Savill, 1995, plus our own unpublished observations). More importantly, a recent report suggested that the initial cytokine exposure monocyte/macrophages receive "programs" subsequent responses to further stimuli such as nitric oxide production and uptake of apoptotic cells (Erwig *et al.*, 1998). DX-treatment from 0-24 hrs following isolation induced a significant phagocytic response, which with certain donors was equivalent to levels of engulfment obtained with 5-days DX-treatment. However in other experiments induction of increased phagocytic capacity was not as evident after removal of glucocorticoids from culture medium As stated previously it is likely that autologous serum contains a number of cytokines and endogenous glucocorticoids which could be variable between donors depending on their immunological status, stress levels, and hormonal balance, and in the absence of glucocorticoids, may alter the macrophage phagocytic response (see also chapter 7).

Erwig *et al.* (1999) demonstrated that uptake of apoptotic neutrophils by rat bone marrow derived macrophages, attenuated further phagocytosis of apoptotic cells on repeated challenge up to 48hrs later, via a mechanism involving $\alpha_v\beta_3$, $\alpha_6\beta_1$ and $\alpha_1\beta_2$.. The number of apoptotic particles cleared by macrophages during the culture period

could also potentially alter the final phagocytic readout. Interestingly 5-day DX treatment significantly decreased the expression of β_1-integrin subunits, in addition to a small decrease in expression of β_3 and $\alpha_v\beta_3$. Decrease in integrin expression levels may partially attenuate intracellular signalling in response to integrin engagement which prevent phagocytosis of apoptotic cells on re-challenge. Uptake of apoptotic cells present in the culture during monocyte/macrophage differentiation may influence phagocytic capability in untreated but not DX-treated cultures, contributing to glucocorticoid augmented phagocytosis. More importantly, the capacity to phagocytose particles on repeated rechallenge implies that the consequences of DX modulation of macrophage phagocytosis in vivo would be far more profound.

In order to reduce intra-experimental variability it would be desirable to reduce lymphocyte contamination by isolation of pure monocyte cultures, normalising serum supplements or employing serum replacements. Highly pure monocytes can be prepared by negative selection using magnetically tagged haptenated anti-lymphocyte mAb and anti-hapten magnetic particles (Macs system, Mitenyi Biotech and Dynabead kits). These techniques are now routinely used for the preparation of monocytes for cell death studies. However Macs isolated monocytes display a slightly altered phenotype after monocyte/macrophage differentiation compared to monocytes isolated by adherent culture, with increased macrophage cell loss after five days in culture. This suggests that although the presence of lymphocytes and lymphocyte cytokines may affect the phagocytic readout, they also facilitate monocyte/macrophage differentiation and survival (Lopez *et al.*, 1993). Alternative serum supplements such as pooled human AB or FCS, have also been explored, but result in reduced viability when compared to autologous serum. An alternative culture system employed in the laboratory is the use of a serum free culture medium, X-vivo, that has recently been made commercially available. Preliminary experiments suggest culture in X-vivo induces macrophage differentiation resulting in a similar phenotype to that obtained by culture with autologous serum, and may therefore be advantageous for further characterisation of the influence of cytokines and other mediators on macrophage phagocytosis.

Dexamethasone acts via the glucocorticoid receptor and requires both transactivation and transrepression functions

Although inclusion of the glucocorticoid receptor antagonist RU38486 confirmed that dexamethasone augmented phagocytosis was mediated via the classical glucocorticoid receptor mediated pathway, we were unable to directly test whether this effect was protein synthesis dependent. Glucocorticoids can both activate and repress gene expression. The acquisition of phagocytic capability for apoptotic cells during monocyte to macrophage differentiation may be due to the expression of cell surface molecules or intracellular components that facilitate the uptake of apoptotic particles. Conversely loss of negative regulators of phagocytosis may occur during monocyte-macrophage maturation. Time course experiments demonstrated that steroid exposure during the first 24hrs of maturation was sufficient to generate a highly phagocytic macrophage phenotype, however inclusion of 5μM cycloheximide (CHX) in the culture media during this period resulted in the death of monocyte populations, as assessed by Hoechst staining (results not shown). Inclusion of CHX later during culture resulted in reduced growth, maturation and viability of both control and DX-treated populations, which was reflected in decreased levels of phagocytosis, compared with non CHX treated macrophages (results not shown). We therefore employed the use of synthetic glucocorticoid analogues, previously characterised to have a predominately transactivational (ZK57740, ZK77945) or transrepressional (RU24782, RU24858) activity (Heck *et al.*, 1997; Vayssiere *et al.*, 1997), in order to characterise the phenomenon at the level of the glucocorticoid receptor. As detailed in figure 5 both transrepressors, and one transactivator (ZK77945) were able to augment phagocytosis consistently above control at 1μM, but never to the same extent as DX. In previous studies the transrepressing compounds RU24782 and RU24858 retained a residual transactivating capacity at higher concentrations. At 1μM RU24782 and RU24858 retained 37% and 25% the transactivating capacity of DX (as measured by the transactivation of a GRE coupled CAT reporter construct in transfected HeLa cells), but have similar transrepressing activity as DX (Vayssiere *et al.*, 1997). Similarly the transactivating ZK- compounds retained residual transrepressing activity. Northern

analysis of collagenase I mRNA expression in TPA treated HeLa cells after treatment with 100nM of the ZK compounds demonstrated that ZK57740 and ZK77945 retained repressional activity of about 50% that of DX (Heck *et al.*, 1997). Northern blots demonstrating the induction of metallothionein IIa mRNA expression after steroid treatment of HeLa cells showed ZK77945 to be a more potent transactivator than ZK57740 (approximately75% compared to 30% of DX activity) (Heck *et al.*, 1997). The failure to augment phagocytosis with ZK57740 could therefore be accountable by a weaker transactivating activity and low transrepressing activity. Interestingly, ABC transporters such as MDR1A can expel synthetic glucocorticoids from within leukocytes with the resulting intracellular concentration lower than that applied in the culture medium (Schinkel *et al.*, 1994; Ueda *et al.*, 1992). Therefore in our culture system extracellular concentrations of ZK57740 may be required to be in excess of 10μM to induce an effect similar to ZK77945. A number of transcription factors implicated in monocyte/macrophage maturation can also be modulated by glucocorticoids, for example C/EBPβ and AP-1 (Gotoh *et al.*, 1997; Herrlich, 2001). Taken together this data may indicate a requirement for both the activation and repression of specific proteins for the induction of a highly phagocytic phenotype. In the advent of new molecular techniques such as array analysis, gene profiling may allow a full description of the molecular changes occurring during monocyte/macrophage differentiation in the presence of glucocorticoids. If we were able to determine if the pro-phagocytic effects were due to the transactivational, or transrepressional activity of glucocorticoids, it may be possible with the use of synthetic steroids to reduce some of the negative side effects of corticosteroids without compromising the beneficial actions.

DX augmented phagocytosis employs multiple receptors

The predominance for utilisation of a specific molecular pathway for uptake of apoptotic cells is not constant between different cell types and can be influenced by certain stimuli. Previous reports have demonstrated that stimulation of human monocyte derived macrophages and murine bone marrow derived macrophages with digestible

particles such as β-glucan, induces a switch from uptake via the $\alpha_v\beta_3$/CD36/TSP pathway, inhibitable with RGDS and anti-$\alpha_v\beta_3$ and antiCD36 mAb, to PS receptor uptake, inhibitable by phospho-L-serine and PS-containing liposomes. DX has been reported to upregulate β-glucan receptor expression, and may further facilitate a switch between uptake pathways on stimulation. In human monocyte/macrophages the switch between phagocytosis mechanisms is accompanied by a down regulation in $\alpha_v\beta_3$ expression, but maintenance of CD36 expression. It is proposed that CD36 either binds PS or facilitates PS-receptor function (Fadok *et al.*, 1998b; Tait and Smith, 1999). In DX treated monocyte/macrophages there was a small decrease in expression of β_3 and $\alpha_v\beta_3$ moieties, and a decrease in binding of the pan CD36 recognising mAb IVC7 (58.1±8.1% of control). However, only a slight change in the CD36 epitope recognised by smϕ (86.6±20% of control) was observed following DX treatment. DX has been well characterised to down modulate pro-inflammatory responses elicited by stimulated immune cells, and it was recently demonstrated that engagement of CD36 by oxLDL but not apoptotic cells induced the release of H_2O_2. The domain of CD36 which mediates PS binding has not been characterised, however it would be intriguing to speculate that it corresponded to the smϕ epitope, and that changes in CD36 conformation, glycosylation or isoform expression could induce a switch between $\alpha_v\beta_3$/CD36/TSP, and PS-receptor mediated uptake pathways.

Assessment of the role of several pathways in glucocorticoid-treated monocyte/macrophages by analysis of receptor expression and functional inhibitor studies failed to define one single pathway as being dominant, suggesting redundancy within the system. This is most likely due to DX "programming" the utilisation of multiple pathways. However, we must consider the validity of the reagents used for receptor blocking studies. Function of competitive ligands such as PLS and fucoidan has been verified in our systems by blocking Annexin V binding to apoptotic neutrophils and inhibition of macrophage uptake of acetylated-LDL. Although the binding affinities may be high enough to validate their function in these assays, it may not be sufficient to

compete with multivalent ligands present on the apoptotic cell surface. The use of higher concentrations may overcome this but would lead to undesirable non-specific effects. One approach to address this problem would be to use multivalent soluble ligands such as lipid microspheres for the blockade of PS-receptor function (Fadok *et al.*, 2000; Tait and Smith, 1999). Another consideration relating to antibody studies would be that crosslinking of receptors (including Fc) by "blocking" mAb or $F(ab')_2$ may induce a number of intracellular signalling pathways which may inhibit phagocytosis independently of the receptor engaged. Use of Fab' fragments, for example as employed in studies on the role of CD14 in phagocytosis of apoptotic cells (Flora and Gregory, 1994) would confirm this.

An alternative approach would be to exploit the specific genetic knockout of phagocytic receptors. However, these studies may also provide equivocal results due to redundancy for clearance of apoptotic cells in healthy pathogen-free animals. For example thrombospondin-1 (TSP-1) knockout mice showed increased susceptibility to pneumonia and changes in lung homeostasis which were due to the broader effects of TSP-1, its ability to activate the latent form of transforming growth factor-β (TGF-β), rather than a specific fault in clearance of apoptotic cells from the absence of TSP-1 (Crawford et al., 1998). Deletion of the class A scavenger receptor produced profound effects on phagocytosis of apoptotic thymocytes *in vitro*, but did not significantly alter the structure of the thymus or the frequency of apoptotic cells present (Platt et al., 1999; Platt et al., 2000). In contrast, studies of patients with systemic lupus erythmematosus (SLE), demonstrated high levels of circulating auto-antibodies, and an increase in the number of apoptotic cells in the skin and blood, due to deficiencies in complement proteins. This prompted the subsequent generation of a C1q deficient mouse which defined a role for complement opsonization in the uptake of apoptotic cells via the C1q receptor. C1q-/- mice develop glomerulonephritis associated with the increased incidence of multiple apoptotic cells in the kidney and a lupus-like disease (Botto et al., 1998). Deposition of C3bi on apoptotic cells has also been implicated in apoptotic cell uptake *in vitro*, however $CD11b^{-/-}$ and $CD18^{-/-}$ mice which lack the CR3 receptor do not

appear to have a defect in clearance of apoptotic cells (Ren *et al.*, 2001). Furthermore macrophages prepared from patients with leukocyte adhesion deficiency (LAD) a condition caused by CD18 deficiency, does not effect apoptotic cell uptake (Davies *et al.*, 1991). A role for the C1q and CR3/CR4 receptors in DX augmented phagocytosis was not specifically assessed in this study. However, macrophages and aged neutrophils are washed out of serum containing medium before assessment of phagocytosis in the absence of serum. Given the short half-life of complement components, participation in the uptake of apoptotic cells by DX treated macrophages may not be apparent in our model. Interestingly analysis of mice with defective Mer, a receptor tyrosine kinase, not previously linked to phagocytosis of apoptotic cells, revealed a role for this protein in the uptake of effete cells. Mice demonstrated an increased number of nuclear autoantibodies, further linking defective clearance with induction of autoimmunity (Scott et al., 2001).

Masking or preventing the disclosure of uptake cues on the apoptotic cell surface could alternately offer a system to define target/phagocyte interactions. Fadok and colleagues. recently demonstrated that depletion of polyamines with difluoromethylornithine (DMFO) in HL-60 cells prior to induction of apoptosis by UV irradiation inhibited uptake by macrophages (Bratton *et al.*, 1999), suggesting a definitive role for PS in the recognition of apoptotic cells. However Cocco and Ucker (2001) showed that PS exposure was not a specific marker of apoptotic cell death as it could also mediate the uptake of necrotic cells. Further cell surface changes may be vital not only for successful engulfment but also for the release of anti- as opposed to pro-inflammatory mediators associated with apoptotic particle engulfment. Therefore redundancy not only at the level of the phagocytic receptor, but also in apoptotic cell motifs must be considered when interpreting data from inhibition studies.

An alternative to specific genetic deletion of putative receptors to define a role in phagocytosis, is their specific over-expression in cell lines previously deficient as phagocytes. Such studies have included transfection of CD36 into Bowes melanoma

cells and COS-7 cell line cells, which conferred a phagocytic phenotype (Ren *et al.*, 1995). However, such studies may be limited to quantifying binding of apoptotic particles due to the lack of other phagocytic machinery required for uptake. Such studies are valuable, but care must be taken not to over-interpret data which does not distinguish between a receptor facilitating uptake by specifically tethering the apoptotic particle, as opposed to engagement of receptors initiating engulfment.

Taken together these results suggest that human peripheral blood monocyte/macrophages employ a number of phagocytic uptake mechanisms, including the PS-receptor without the requirement for stimulation with digestible particles. I would suggest that the utilisation of multiple pathways may contribute to the highly phagocytic phenotype generated after exposure to DX.

DX treatment alters macrophage phagocytic machinery

Cross-linking of CD44 on the macrophage cell surface induces an increase in phagocytic capacity, which is specific for apoptotic neutrophils, and was able to further augment phagocytosis in DX treated monocyte/macrophages. Although uptake of other apoptotic targets after 5-day DX treatment was not assessed in this study, short-term glucocorticoid treatment (24hrs prior to the phagocytosis assay) potentiated phagocytosis of apoptotic eosinophils and lymphocytes (Liu *et al.*, 1999). We would therefore predict that glucocorticoids induce an increase in phagocytic capability for multiple targets, in a manner distinct from the selective mechanisms employed by CD44 cross-linking. Little is known about how CD44 cross-linking induces phagocytosis. Preliminary evidence suggests that bone marrow derived macrophages from CD44-/- mice have higher basal phagocytosis levels than their wild type counterparts (I Dransfield personal communication), suggesting that CD44 may act as a negative regulator of apoptotic cell uptake, possibly due to its highly glycosylated (and therefore charged) nature. Crosslinking would sequester CD44 allowing areas of the cell membrane to participate in particle engulfment. Downregulation of CD44 in response to

DX treatment, measured by pan CD44 mAb, and the v3 epitope, could therefore contribute to glucocorticoid-augmentation of macrophage phagocytosis. However after CD44 crosslinking a similar relative augmentation of phagocytosis of apoptotic neutrophils was seen in 5-day DX treated macrophages compared to untreated macrophages. Additionally, the effect of DX on expression of individual splice variants, which may be specific for the induction of phagocytosis on crosslinking has not been addressed. Assessment of the phagocytic capability of CD44-/- macrophages exposed to glucocorticoids may provide a clearer insight. Intracellularly, CD44 has been shown to interact with ERM (ezrin/radixin/moesin) proteins, important mediators of cortical actin structure and Tiam1 a GEF for Rac, promoting extension of cellular processes (Oliferenko *et al.*, 2000). Research in our laboratory has previously demonstrated the importance of adhesion and cytoskeletal dynamics in modulating macrophage phagocytic capacity (McCutcheon *et al.*, 1998; Rossi *et al.*, 1998). For example, blockade of actin or tubulin dynamics with cytochalasin or nocodazole prevent phagocytosis of apoptotic cells and opsonized particles, as do inhibitors of tyrosine kinase activity (e.g. genestein), which prevent the turnover of podosomes and other focal contacts. Conversely, disruption of adhesive contacts by elevation of cellular cAMP inhibits phagocytosis of apoptotic cells. Analysis of potential changes in adhesion signalling and cytoskeletal organisation after DX treatment may provide further clues to establish how glucocorticoids promote a highly phagocytic macrophage phenotype.

CHAPTER 4: CHANGES IN MACROPHAGE ADHESION SIGNALLING IN RESPONSE TO GLUCOCORTICOIDS

Introduction

Phagocytosis requires the local rearrangement of the actin cytoskeleton. Therefore it is not surprising that cell adhesion and adhesion signalling molecules which are able to modulate actin dynamics have been strongly implicated as regulators of macrophage phagocytic capability. Disruption of cytoskeletal and adhesion contacts in monocyte-derived macrophages by elevation of intracellular cAMP inhibits phagocytosis of apoptotic cells (Rossi *et al.*, 1998). In contrast, ligation of CD44 (Hart *et al.*, 1997) or adhesion to fibronectin (McCutcheon *et al.*, 1998) results in a rapid and dramatic augmentation of apoptotic cell uptake. The mechanisms underlying this association remain to be determined, however it is notable that many of the structural components and regulators of adhesive contacts are also associated with phagocytic machinery, for example WASp and the Rho family of GTPases (Allen and Aderem, 1996; Burns *et al.*, 2001; Caron and Hall, 1998; Leverrier *et al.*, 2001; Linder *et al.*, 1999; Ory *et al.*, 2000). Substances which perturb actin dynamics induce the disassembly of podosomes and other adhesive contacts within macrophages, and prevent phagocytosis *per se* (Allen and Aderem, 1996; Newman *et al.*, 1991). Additionally tyrosine kinase inhibitors that also alter adhesive contacts, prevent phagocytosis of apoptotic cells and IgG-opsonized particles. Complement receptor-mediated uptake however, appears insensitive to tyrosine kinase inhibition (Allen and Aderem, 1996; Newman *et al.*, 1991), suggesting that certain cytoskeletal components may be selectively involved in the control of phagocytic processes.

Similarities between phagocytosis and adhesion/migration have been further demonstrated at an ultrastructural level using electron microscopy. FcR-phagocytosis and apoptotic cell uptake appears to be mediated by extension of membrane processes similar to lamellipodia, around the particle (Giles *et al.*, 2000; Griffin *et al.*, 1975b; Leverrier *et al.*, 2001), whereas complement-opsonized particles appear to sink into the phagocyte surface (Griffin *et al.*, 1975a; Kaplan,

1977). Recycling of membrane in lamellipodia at the leading edge of the cell, is thought to be vital for cell migration (Bretscher and Aguado-Velasco, 1998). Likewise inhibition of recycling endosomes with tetanus toxin or by blockade of PI 3-Kinase activity, prevents phagocytosis of IgG opsonized particles, and apoptotic cells (Araki *et al.*, 1996; Bajno *et al.*, 2000; Cox *et al.*, 1999; Leverrier and Ridley, 2001; Ninomiya *et al.*, 1994).

Integrin ligation induces the formation of a multi-protein complex involving the adapter proteins Crk, p130Cas, and DOCK180, and the guanine nucleotide exchange factor C3G (Hasegawa *et al.*, 1996; Kirsch *et al.*, 1998; O'Neill *et al.*, 2000; Sakai *et al.*, 1994). Assembly and membrane localisation of this complex activates the GTPases Rac (Kiyokawa *et al.*, 1998a; Kiyokawa *et al.*, 1998b) and Rho (Altun-Gultekin *et al.*, 1998), inducing membrane ruffling and lamellipodia formation (Allen *et al.*, 1997). Studies in mammalian cells by Albert *et al.* suggest that ligation of $\alpha_v\beta_5$ on the surface of dendritic cells by adhesion to ECM, or engagement of an apoptotic particle, induces formation of the DOCK180/Crk/p130cas complex, subsequent Rac activation and uptake of apoptotic cells. Additionally Ravichandran and colleagues have described a close correlation between Crk and DOCK180 association, Rac activation and apoptotic cell engulfment (Tosello-Trampont *et al.*, 2001). The lack of complete inhibition of phagocytosis by soluble ligands or blocking mAb in DX-treated monocyte/macrophages points to functional redundancy at the level of engagement of the apoptotic particle, and raises the possibility that glucocorticoids modulate phagocytic potential through regulation of these intracellular signalling pathways. Several reports suggest that short term (48hr) glucocorticoid treatment of monocyte/macrophages can augment the uptake of a number of particles including myelin and *Staphylococcus aureous* (van der Goes *et al.*, 2000). 5-day DX treated monocyte/macrophages appear to employ multiple pathways to ingest apoptotic cells, however the effect of DX-treatment on general phagocytic function has not been addressed. Lui *et al.* (2000) demonstrated that short term DX treatment (24hrs) did not augment FcR-mediated uptake of opsonized erythrocytes. Assessment of phagocytosis of alternative particles, analysis of cytoskeletal organisation and the formation of adhesion processes after DX treatment

may provide further clues as to the key molecules regulating the phagocytosis of apoptotic cells.

Results

DX augments phagocytic capacity per se

In order to investigate whether long term DX-treatment altered macrophage phagocytic capacity generally we assessed uptake of erythrocytes after high and low density opsonization with IgG. Erythrocytes were pre-incubated with polyclonal anti-erythrocyte surface membrane antibody to give either a 1:100 (high), or a 1:2000 (low) opsonization density. In a 20 minute assay we found an increase in uptake of highly opsonized (1:100) RBC after DX treatment from 48.2±6.7% (control) to 61.0±9.4 (DX) (mean ±SEM, n = 6) (Fig1a). However, when erythrocytes were opsonized at lower concentrations (1:2000) a significant effect of glucocorticoids on FcR-mediated phagocytosis became apparent, with 27.9±4.9% of control populations ingesting particles compared to 57.5±9.4% after DX treatment (mean ±SEM, n = 7, $p < 0.05$). Although low opsonization represented a limiting factor for the uptake of particles by untreated macrophages, phagocytosis by DX-treated monocyte/macrophages was comparable between high and low opsonization (fig 1a). Uptake of whole zymosan or complement opsonized zymosan particles, ingested via β_2-glucan and complement receptors respectively also appeared to exhibit differential trends for untreated and DX-treated cells. Interestingly uptake of whole zymosan appeared to be slightly elevated with DX treatment: 61.9±3.1%, compared to 54.7±5.1% by untreated populations (mean±SEM, n = 4). In contrast, preliminary data suggested that 5-day DX treatment produced a slight inhibition of phagocytosis of complement opsonized zymosan (untreated: 72.5%, DX-treated: 65.3%, mean, n=2). However, further experiments are required in order to see if these trends are statistically significant. Interestingly, although DX-treated cells were more proficient at phagocytosing opsonized particles, expression of the high affinity Fc-receptor CD64 (FcRγI) was decreased after steroid treatment (See chapter 7). The lower affinity receptors FcγRII (CD32) and FcγRIII (CD16) also contribute to the

phagocytic capacity for IgG opsonized particles. Expression of CD32 was not assessed, however expression of CD16 was slightly elevated after DX treatment. Surface expression does not always reflect function, suggesting that glucocorticoid treatment may affect intracellular components common to the ingestion of both opsonized particles and apoptotic cells.

Inhibitors of phosphatidyl-3-kinase block DX-treated macrophage phagocytosis of IgG-opsonized particles, and apoptotic cells

PI 3-kinase is required for membrane recycling during extension of cellular processes (Cox *et al.*, 1999). Inhibition of PI 3-kinase activity with either wortmannin or the specific inhibitor LY294002 has been previously shown to inhibit the uptake of both IgG-opsonized particles and apoptotic cells (Araki *et al.*, 1996; Cox *et al.*, 1999; Leverrier and Ridley, 2001). To assess whether 5-day DX treated monocyte/macrophages employed a similar signalling mechanism for the uptake of apoptotic cells as untreated macrophages, we assessed phagocytosis in the presence of the PI 3-Kinase inhibitor LY294002 (fig. 2a). Pre-treatment of monocyte/macrophages with up to 100μM LY294002 for 1hr prior to the phagocytosis assay, inhibited uptake of apoptotic neutrophils in both untreated and DX-treated cultures from 10.7±2.0% and 41.3±11.7% to 2.0±0.5% and 7.9±3.8% respectively (mean ±SEM, n=4). Preliminary experiments suggested that LY294002 also produced a slight inhibition of opsonized erythrocyte phagocytosis (fig. 2b). In preliminary experiments 100μM LY294002 reduced ingestion of low opsonized erythrocytes (as described for figure 1), from 17.5% to 12.6% (untreated) and 39.9% to 27.1%, (DX-treated) (n=2). Failure to produce such a profound inhibition as seen with apoptotic cells may reflect the strength of signal mediating phagocytosis, or alternatively an effect relating to particle size. In support of the latter suggestion, PI 3-kinase activity did not appear to be required for phagocytosis of whole or complement-opsonized zymosan particles (data not shown), which are ingested via a phagocytic pit rather than extension of membrane processes as in FcR-mediated uptake. Together these data suggest that DX-treated monocyte/macrophages employ the same fundamental PI 3-kinase dependent pathways for engulfment as untreated monocyte/macrophages, however the efficiency of either recognition by cell surface

receptors, or recruitment of intracellular components is augmented with glucocorticoids.

DX alters macrophage morphology

Examination of monocyte/macrophage cultures by phase contrast revealed distinct morphological changes after maturation in the presence of glucocorticoids. Untreated macrophages were composed of a morphologically heterogeneous population of cells, with multinucleated giant cells (MNGCs), "spread", apparently motile cells with evidence of membrane ruffling, "rounded" less spread cells and the occasional dendritic cell-like morphologies (fig. 3a). In contrast treatment of freshly isolated monocytes with DX for 5 days induced a homogenous population of cells, as described in chapter 3, with increased numbers of small "rounded" cells, reduced numbers of "spread" macrophages and an absence of multinuclear cells (fig. 3b). Examination of macrophage morphology using scanning electron microscopy further illustrated the decreased cell spreading in DX-treated cultures compared to control (fig. 3d,e). However the "rounded" DX-treated macrophages were attached, with ruffled membranes and filopodial processes. Interestingly in untreated macrophage cultures it was apparent that the phagocytic capacity was related to the morphological appearance of macrophages. MNGC were consistently poor phagocytes, with less that 1% of cells phagocytosing, whereas smaller, more rounded monocyte/macrophages were more phagocytic (data not shown). The observation would be consistent with DX treatment "re-programming" monocyte/macrophage morphology and adhesion in concert with augmented phagocytic capability. The concept of re-programming of monocyte-macrophage differentiation and behaviour will be further explored in chapters 7 and 5 respectively.

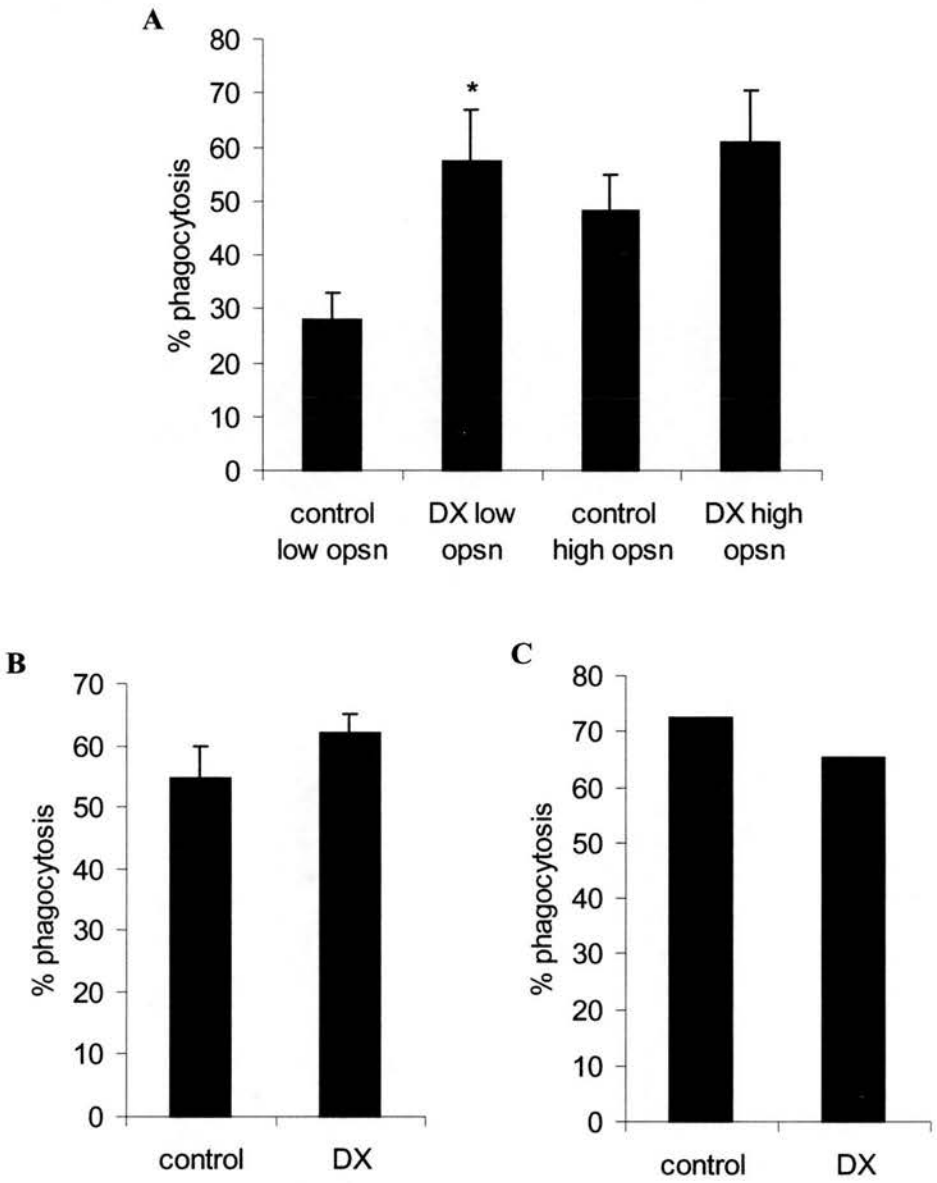


Figure 1. DX augments phagocytosis of IgG but not complement opsonized particles
Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM dexamethasone for 5 days, and macrophage phagocytic capacity assessed. **A** Macrophage monolayers were overlaid with erythrocytes opsonized by either high or low mAb concentration, and levels of uptake, assessed microscopically after a 20 minute assay, as previously described. Mean phagocytosis ±SEM, $n = 6$. **B** Macrophage monolayers were exposed to boiled zymosan, (mean phagocytosis ±SEM, $n = 4$), or **C** complement opsonized zymosan (mean phagocytosis $n = 2$) over a twenty minute period. Zymosan uptake was quantified by removal of monocyte/macrophages with trypsin EDTA, and analysis of cytospin preparations. (*, $p < 0.05$ vs. control values, Wilcoxon matched pairs test).

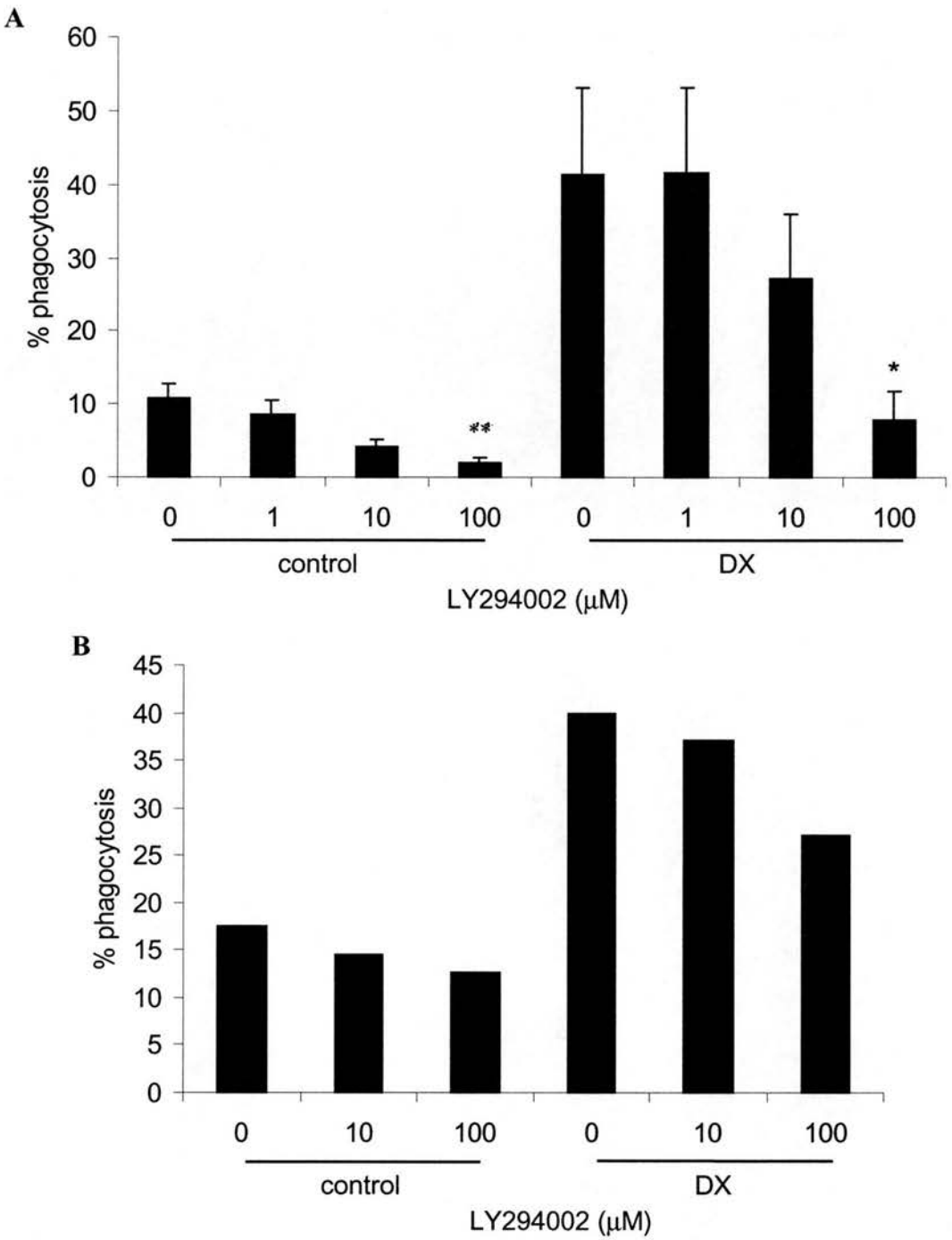


Figure 2. Inhibitors of PI-3 Kinase block phagocytosis of apoptotic cells and IgG-opsonized particles

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM dexamethasone for 5 days. Macrophages were pre-incubated with varying concentrations of LY294002 for 1 hr, prior to assessment of phagocytosis of **A** apoptotic cells, mean phagocytosis ±SEM, $n = 4$, and **B** IgG-opsonized erythrocytes, mean phagocytosis, $n = 2$. Levels of phagocytosis were assessed as previously described (*, $p < 0.05$; **, $p < 0.01$ vs. non-LY294002 treated values, Friedman Test with post test).

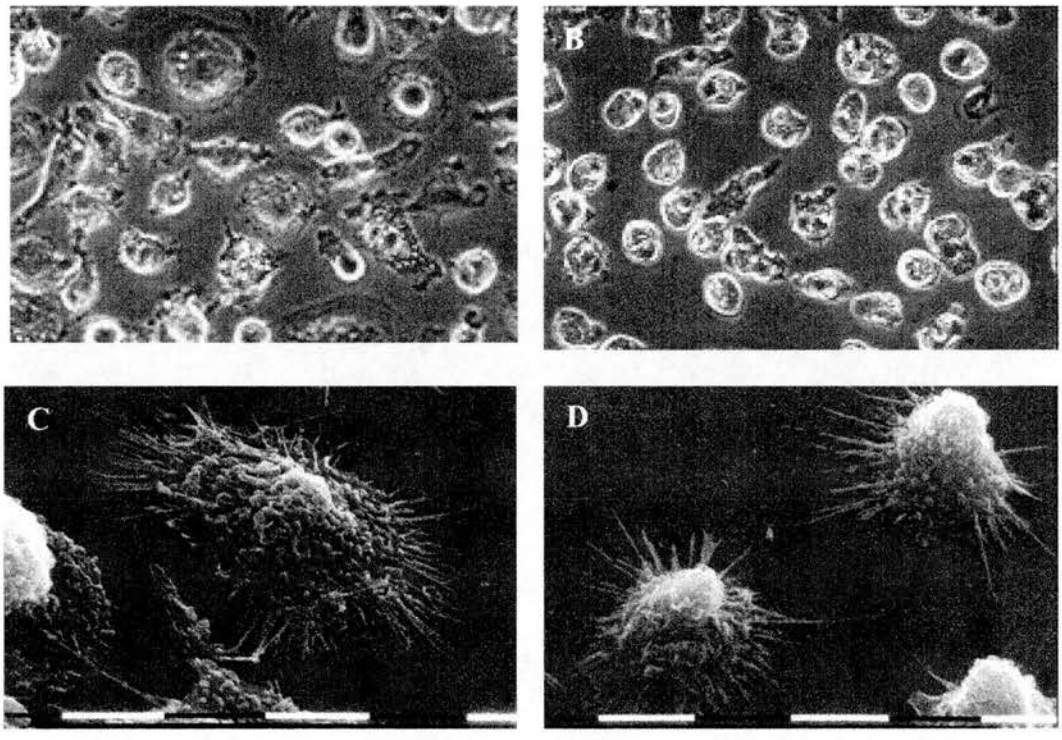


Figure 3. Effect of DX on macrophage morphology
Adherent peripheral blood monocytes were cultured for 5 days in the presence or absence of 1μM DX on either tissue culture treated plastic (A, B) or glass coverslips (C, D). Morphology of untreated (A, C), and DX treated (B, D) were assessed by phase contrast microscopy (A, B) (x40 objective), and scanning electron microscopy (C, D) (bar equals 10μm). Note the uniform appearance of DX-treated cells by phase contrast microscopy, which is also observed in electron micrographs.

Glucocorticoids alter macrophage cytoskeletal organisation

The distinct morphological appearance of DX-treated macrophages shown in figure 3, suggested that control of adhesion was altered following DX-treatment. In order to assess the effect of DX programming on the formation of macrophage adhesive processes, we examined the extent of focal contact and podosome formation in untreated and DX treated cells by immunofluorescent staining of actin and paxillin, proteins also recruited to phagocytic cups during FcR-mediated, and apoptotic cell phagocytosis. Monocyte/macrophages were matured for 5-days +/- 1μM DX on glass coverslips. Culture on glass did not appear to alter macrophage morphology and enabled examination of cells at higher magnification. Cells were fixed in paraformaladehyde, permeabilised using NP-40, actin visualised by binding of rhodamine-coupled phalloidin, and paxillin localised with an anti-paxillin mAb. Untreated cells showed punctate (podosome-like) staining of actin (fig. 4d) with concentric association of paxillin, representing sites of cell-substratum contact (fig 4e). Paxillin was also observed towards the cell periphery coincident with the localisation of other proteins associated with adhesion (vinculin and tyrosine phosphorylated proteins) previously demonstrated in the laboratory (Rossi *et al.*, 1998) and consistent with the adherent phenotype of macrophages observed under phase microscopy. In contrast, DX-treated cells showed an absence of podosomes and a reduction of focal contacts at the cell extremity. Paxillin was distributed diffusely throughout the cytoplasm, and actin organised irregularly around the periphery of the cell. Interestingly MNGCs present in untreated cultures contained an extensive number of adhesive contacts. We could speculate that this might hinder membrane extension and recycling of proteins required for the engulfment of particles thereby reducing their phagocytic capacity as described in the previous section.

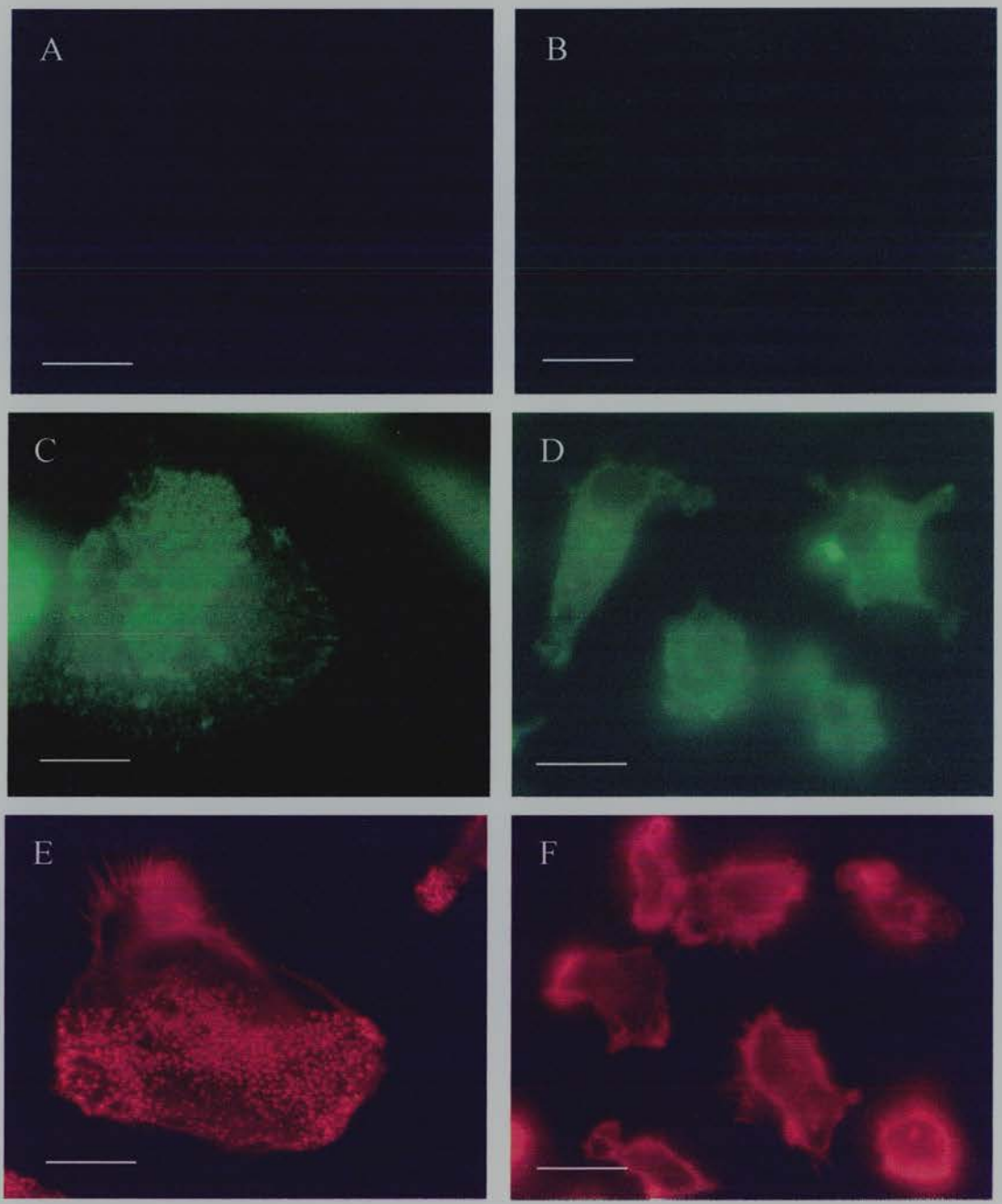


Figure 4. Effects of dexamethasone on localisation of actin and paxillin in macrophages. Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1μM DX. Localisation of paxillin and actin was determined using either anti-paxillin mAb (C, D) or rhodamine phalloidin (E, F) together with fluorescence microscopy. Staining observed with control mAb is shown in panels A and B. These representative micrographs (n=3) illustrate typical punctate actin staining of "contact sites" similar to podosomes in control macrophages that are absent in DX-treated macrophages. Paxillin is also localised to smaller "focal adhesion-like" structures near the cell periphery in control macrophages, that are less well-defined in dexamethasone-treated macrophages. Scale bar equals 20μm

DX disrupts components of monocyte/macrophage adhesion signalling: Changes in phosphorylation of paxillin and Pyk2

Formation of podosomes and focal contacts is a very dynamic process (Chellaiah *et al.*, 2000; Ory *et al.*, 2000). Disassembly and turnover of podosomes and focal contacts is thought to occur after changes in phosphorylation of adhesion components or disassembly of actin structures (Fincham and Frame, 1998). To investigate how DX treatment may prevent podosome formation we assessed the phosphorylation state of paxillin and pyk2 by western blot analysis of immunoprecipitated proteins. Monocyte/macrophages were cultured for 5-days +/- 1μM DX in 6-well culture plates as previously described. Monolayers were lysed on the plate (to prevent changes in adhesion signalling components during removal of cells) using an NP-40 based lysis buffer, and paxillin and Pyk2 immunoprecipitated using specific mAb. The resulting protein/antibody complex was separated using SDS-PAGE and analysed by western blotting with either anti-paxillin or Pyk2 mAb, to ensure equal loading, plus an anti-phosphotyrosine mAb, RC-20. Both paxillin and Pyk2 were expressed at equivalent levels in untreated and DX treated populations, however tyrosine phosphorylation of both paxillin and Pyk2 was markedly decreased after DX-maturation (fig. 5a). This correlated directly with loss of podosome structures, as monocyte/macrophages exposed to DX for 24hrs after 4-days maturation, did not show changes in adhesion, or a decrease in paxillin and Pyk2 phosphorylation (fig. 5b).

As recruitment of paxillin to focal contacts is thought to require phosphorylation, it is likely that localisation to nascent phagosomes is also dependent on the production of phosphorylated paxillin. Failure of DX treatment to block paxillin phosphorylation in 4 day differentiated monocyte/macrophages, suggests that DX does not alter kinase/phosphatase activity directly, and we would therefore predict that 5-day DX-matured macrophages would phosphorylate paxillin on stimulation. To assess kinase function, we stimulated DX-treated monocyte/macrophages by re-adherence to plastic, and ligation of β_1-integrins with an activating mAb 12G10. Mononuclear cells were isolated from peripheral blood as previously described, and monocytes purified by MACs isolation. Monocytes were then cultured either in suspension

using Teflon pots, or by adherence to tissue culture plastic for 5-days +/- 1μM DX. After maturation in suspension, monocyte/macrophages were adhered to tissue culture plastic for 1hr, or pelleted, prior to lysis, and paxillin immunoprecipitation analysis as before. Non adherent (pelleted) cells showed no detectable paxillin phosphorylation, and macrophages matured for 5 days by adherent culture demonstrated phosphorylation of paxillin in untreated but not DX treated cultures as shown previously. However, in contrast phosphorylated paxillin was present in both untreated and DX-treated cells cultured in suspension and then re-plated (fig. 6a), suggesting functional kinase activity in the presence of DX. A full phenotypic analysis of suspension cells was not undertaken to ensure DX-programming occurred in non-adherent cells, however DX-treated suspension cells did appear to have reduced expression of p130cas (data not shown), and had increased phagocytic function (I. Dransfield, personal communication).

Ligation of β_1-integrins by cross-linking with monoclonal antibodies or adherence to ECM induces the phosphorylation and localisation of paxillin and Pyk2 to sites of focal contact. Flow cytometric analysis of macrophage phenotype after DX treatment revealed a significant decrease in β_1-integrin expression (chapter 3), which may contribute to the altered adhesive state observed. The induction of phosphorylation of paxillin after macrophage "replating" may reflect the repertoire of receptors employed for initial adhesion, with altered receptor usage during long term culture. In an attempt to directly assess the contribution of β_1-integrins in phosphorylation of both paxillin and Pyk2 after DX treatment, we stimulated cell surface β_1-integrins, with the activating mAb 12G10. Monocyte/macrophages were matured by adherent culture +/- DX as before. Monolayers were incubated with normal rabbit immunoglobulin for 15 min, to block mAb binding to endogenous Fc-receptors, cells were then washed, and incubated with 12G10 for a further 20 min prior to lysis and immunoprecipitation of paxillin and Pyk2 as described before. Ligation of β_1-integrins with 12G10 had no effect on phosphorylation of Pyk2 in either untreated or DX-treated cells, however the mAb induced a small increase of paxillin phosphorylation in untreated monocyte/macrophages (fig 6b). 12G10 was unable however to stimulate paxillin phosphorylation in DX-treated cells, inferring

that changes in adhesion in DX-treated monocyte/macrophages may involve defects in β_1-integrin signalling. Repetition of this experiment using suspension cultured monocyte/macrophages which have reduced integrin ligation and subsequently paxillin phosphorylation, would confirm the effect was due to defective signalling rather than a sequestration of β_1-integrins by adhesion of the cell to its substratum. Ligation of integrins, including β_1, via apoptotic cell engagement or monoclonal antibodies has been reported to inhibit subsequent phagocytosis of apoptotic cells (Erwig *et al.*, 1999). Conversely components of β_1-integrin signalling have been shown to be involved in the engulfment of apoptotic particles (Albert *et al.*, 2000; Tosello-Trampont *et al.*, 2001). Considering the highly phagocytic nature of DX-treated macrophages, we next considered possible modulation of β_1-integrin signalling components in glucocorticoid matured macrophages.

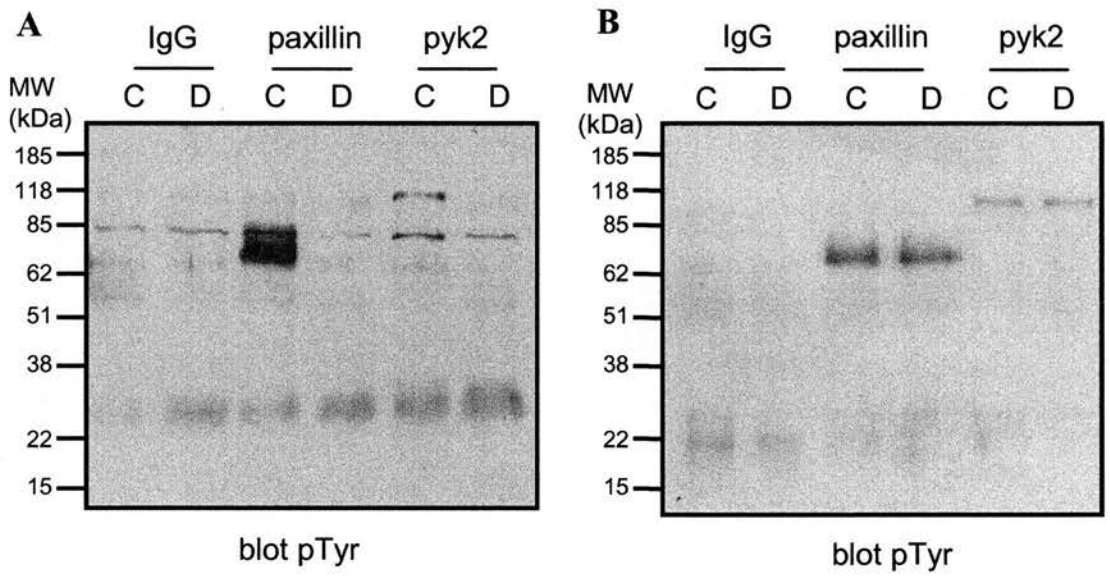


Figure 5. Dexamethasone downregulates phosphorylation of pyk2 and paxillin.
Monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1μM DX, or were exposed to DX for 24 hr after 4 days maturation. Pyk2 and paxillin were immunoprecipitated from cell lysates and protein phosphorylation of immunoprecipitated proteins analysed by SDS PAGE together with Western blotting using the anti-phosphotyrosine mAb RC-20 (1:5000) and enhanced chemiluminescence detection. In this gel, IgG denotes protein phosphorylation patterns associated with a non-binding IgG1 control mAb. The band at ~85kDa, present to some extent in all immunoprecipitates probably represents a "non-specific" component. Lanes labelled (C) are from untreated control macrophages and (D) are from DX-treated macrophages. **A** Macrophages cultured ± DX for 5 days. **B** Macrophages cultured in DX for 24 hr, after 4 days maturation.

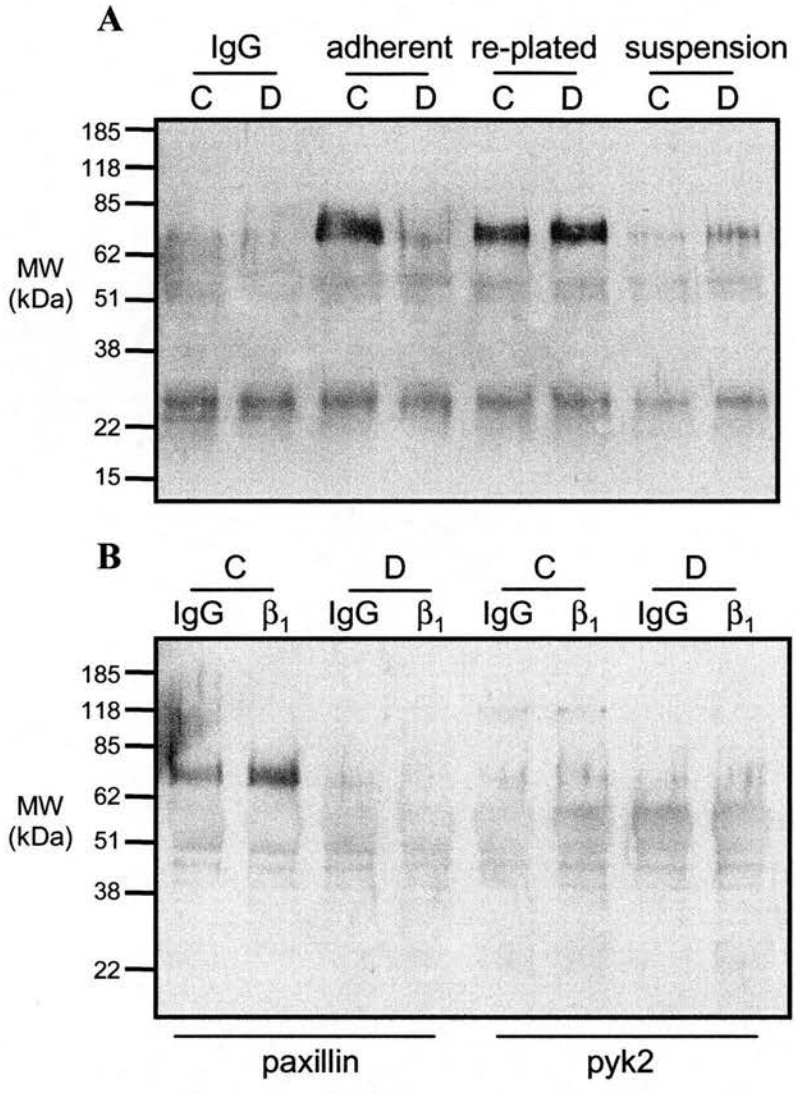


Figure 6. Stimulation of paxillin phosphorylation in DX-treated macrophages
Monocyte/macrophages treated with DX were assessed for phosphorylation of paxillin under various conditions. **A** Peripheral blood monocytes were MACs purified from blood mononuclear cell fraction, and cultured for 5 days in the presence or absence of 1μM DX by either adhesion to tissue culture plastic as described previously, or by suspension in Teflon coated wells. Lysates of adherent cultured macrophages, suspension cultured macrophages adhered to virgin plastic for 1 hr, or pelleted suspension cultured macrophages were assessed for paxillin phosphorylation by immunoprecipitation, SDS-PAGE, and western blotting analysis, as for figure 5. **B** Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1μM dexamethasone as described previously. Monolayers were incubated with normal rabbit IgG for 15 min to block non specific FcR binding, washed, and incubated with either a control IgG, or β_1-activating mAb 12G10, for 30 min, and paxillin and pyk2 phosphorylation assessed as for figure 5. Lanes labelled (C) are from untreated control macrophages and (D) are from DX-treated macrophages. IgG and β_1 denote cells stimulated with control mAb and activating 12G10 mAb respectively.

DX alters components of β_1-integrin signalling

The p130/cas/crk/DOCK180 complex is a major mediator of adhesion signalling, and is recruited to sites of membrane-ECM contact after integrin ligation inducing actin organisation, stress fibre formation, and membrane ruffling via activation of Rho-family GTPases (Altun-Gultekin *et al.*, 1998; Kiyokawa *et al.*, 1998a; Kiyokawa *et al.*, 1998b). Formation of the complex requires phosphorylation of p130cas which then functions as a molecular scaffold, recruiting Crk and DOCK180. Monocyte/macrophages matured for 5 days +/- DX were lysed and p130cas phosphorylation assessed by immunoprecipitation analysis as for paxillin. In these experiments DX treatment appeared to inhibit p130cas phosphorylation (data not shown). However, analysis of whole cell lysates (WCLs) with a mAb specific for p130cas demonstrated that the apparent attenuated phosphorylation was the result of a down-regulation of p130cas expression (fig 7c). A time course of p130cas expression during monocyte-macrophage differentiation in the presence of glucocorticoids revealed that down-regulation was initiated early, after 24-48hrs of DX treatment (results not shown), suggesting changes in p130cas expression are initiated within the "time window" of DX exposure required to induce a highly phagocytic macrophage phenotype (chapter 3). Furthermore, exposure of monocyte/macrophages to DX for 24 hrs after 4 days maturation in the absence of steroids failed to alter p130cas expression (fig 7c), as was seen with paxillin phosphorylation (fig. 5b). Protein levels of CrkII or the haematopoietic cell specific homologue CrkL were unchanged after 5 day DX treatment (fig 7a,b). Technical difficulties prevented us reproducibly immunoblotting DOCK180 (data not shown); therefore semi-quantitative RT-PCR was used to analyse the levels of DOCK180 and the haematopoietic cell specific M-DOCK mRNA transcripts. No differences in mRNA levels were detected after DX treatment, although this does not exclude the possibility that differences may be apparent at the protein level (fig 7d).

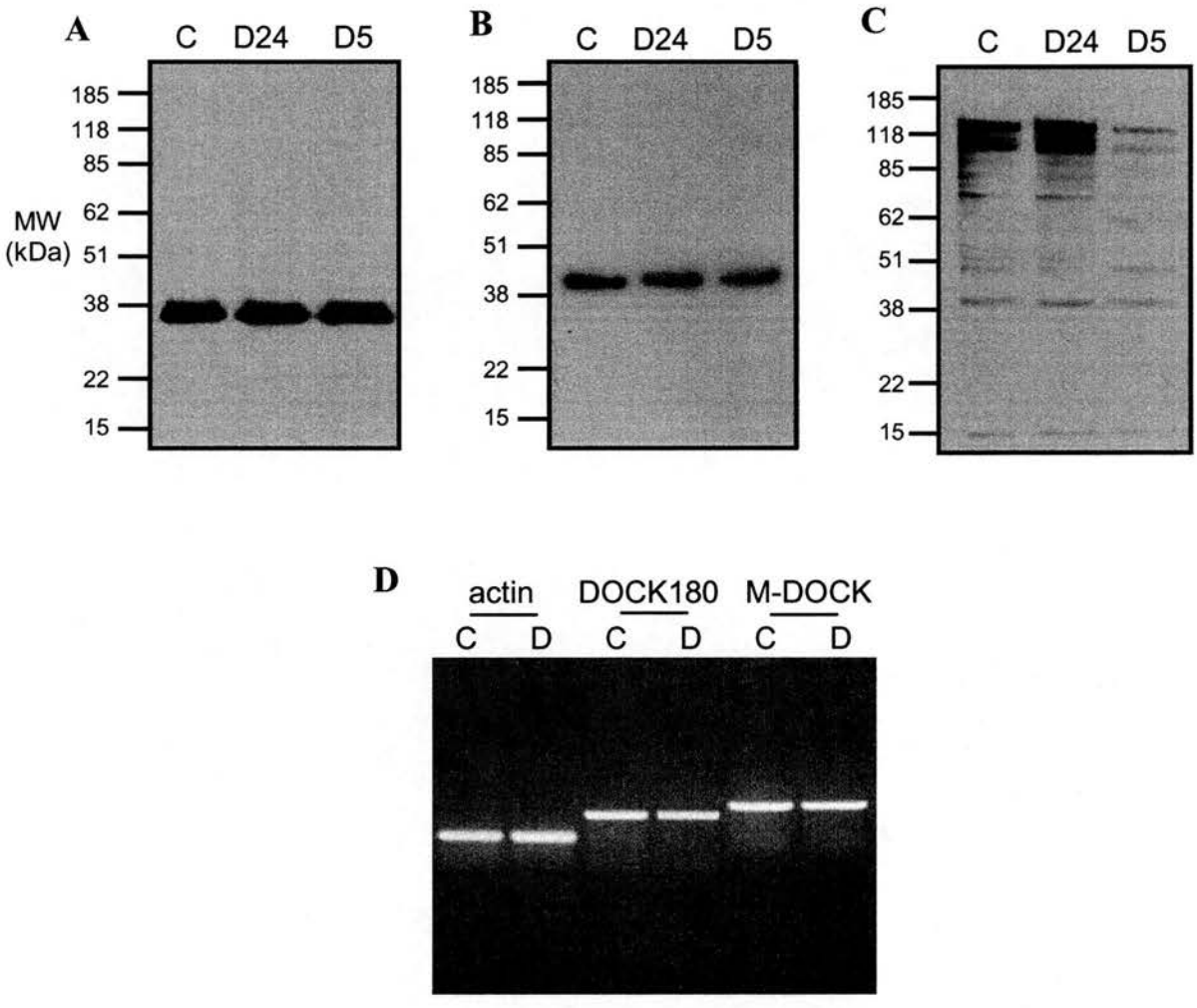


Figure 7. DX alters components of β_1-integrin adhesion signalling
Adherent peripheral blood monocyte-derived macrophages were cultured in the absence (lanes labelled C) or presence of 1μM dexamethasone for 5 days (D5), or 24hrs after 4 days maturation (D24). Macrophage cell lysates were assessed for expression of **A** CrkL (36 kDa) **B** CrkII (40/42 kDa) and **C** p130Cas (130 kDa) by SDS-PAGE and Western blotting. Antibodies used at 1:500, CrkL, CrkII; 1:5000, p130cas. **D** Total RNA from untreated and dexamethasone-treated macrophages were assessed for expression of actin, DOCK180, and M-DOCK/DOCK2 transcripts using RT-PCR with primers described in Materials and Methods.

DX augments Rac activity

The p130cas/crk/DOCK180 complex is required for Rac activation during the formation of adhesion processes in a number of cell types, and phagocytosis of apoptotic particles by dendritic cells and cell lines reconstituted with phagocytic machinery (Albert *et al.*, 2000; Tosello-Trampont *et al.*, 2001). We would predict that the down regulation of p130cas expression may disrupt formation of the crk/DOCK180 complex, compromising Rac activity, and thus correlate with the altered adhesion and cytoskeletal organisation of DX treated cells. However, the highly phagocytic nature of DX-matured cells suggests that Rac is highly active. We therefore evaluated Rac activity directly using a pull-down assay utilising the CRIB domain of PAK (p21 activated kinase) which binds GTP-bound (active) Rac. Monocyte/macrophages matured +/- DX as described previously were lysed and the resulting lysates probed with GST-CRIB fusion protein coupled to Sepharose beads. Levels of active Rac were then quantified by SDS PAGE and western blotting. Both untreated and DX-treated cultures expressed equal amounts of total Rac (quantified by probing whole cell lysates with an anti-Rac mAb), however, untreated control macrophage populations showed little detectable active Rac compared to high levels in DX-treated cultures (fig. 8). Furthermore localisation of total Rac by immunofluorescence demonstrated distinct staining patterns between untreated, and DX-treated macrophages. In untreated cells Rac was localised to membrane ruffles (fig 9c), whereas membrane ruffling was decreased in DX-treated macrophages, with Rac distributed in a polarised manner around certain parts of the cell membrane (fig. 9d).

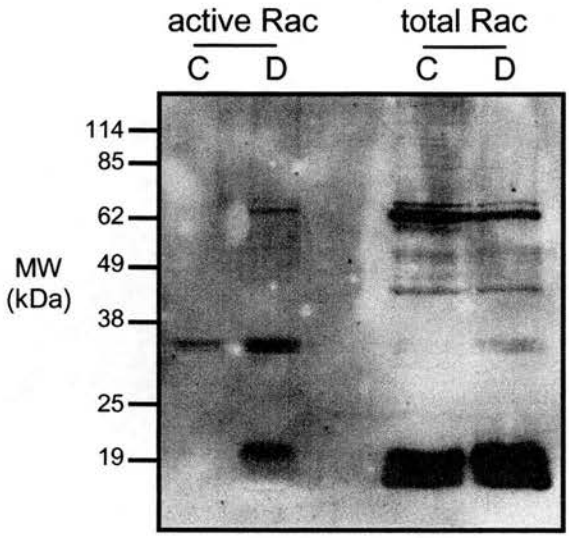


Figure 8. Augmented Rac activity in monocyte/macrophages treated with DX
Adherent peripheral blood monocyte-derived macrophages were cultured in the absence (lanes labelled C) or presence of 1μM dexamethasone for 5 days (D5). Activity of the GTPase Rac in macrophage lysates was assessed using "pull down" assays employing Sepharose coupled with GST-PAK protein. SDS PAGE and Western blotting with a Rac-specific mAb (1:1000) was used to determine levels of Rac in "pull-downs" (Active Rac) and Rac expression levels in whole cell lysates (Total Rac).

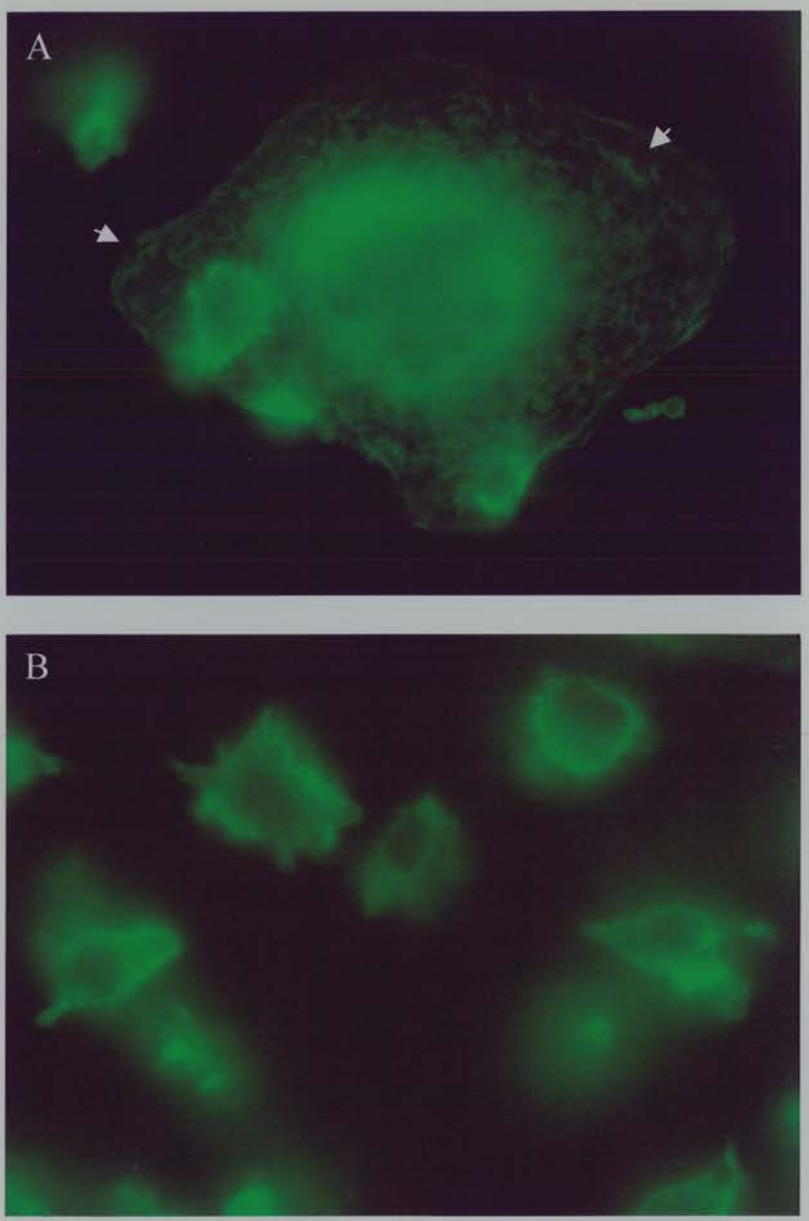


Figure 9. Altered Rac localisation in DX treated macrophages
Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1μM DX. Localisation of Rac was determined using anti-Rac mAb along with fluorescence microscopy. For staining with control mAb see figure 4. **A** control untreated macrophages. **B** DX-treated macrophages. These micrographs demonstrate characteristic Rac localisation to membrane ruffles present in untreated macrophages, indicated by the arrow head. Ruffles are not apparent in DX treated cells, however Rac localisation is polarised across the cell.

DX alters expression of the Rac GEF, Tiam1

The high levels of detectable active Rac would suggest GEF activity may be elevated in DX treated cells. A number of GEFs for Rac have been identified. These include Vav (Crespo *et al.*, 1996; Crespo *et al.*, 1997; Olson *et al.*, 1996), and Tiam1 (Leeuwen *et al.*, 1997; Michiels *et al.*, 1995; Michiels *et al.*, 1997). Increased expression or activity of alternative GEFs could account for the increased Rac activity in DX treated cells. We therefore assessed the expression of Vav, and Tiam1 in macrophage lysates by SDS-PAGE and western blotting. Surprisingly 5-day DX treatment down regulated the expression of Tiam1, but maintained the expression of Vav (fig. 10). Activation of Vav by phosphorylation of specific tyrosine residues promotes Rac binding. Although preliminary immunoprecipitation experiments suggested that DX-treatment decreased Vav phosphorylation and therefore its GEF activity, further work would be required to determine the role of Vav in DX-augmented Rac activity.

Recruitment of alternative adaptor proteins may compensate for loss of p130cas expression and promote DOCK180 function. Changes in cellular localisation of DOCK180 by the engagement of alternative adaptor molecules may also promote Rac activity at membrane sites involved in phagocytosis as opposed to the formation of adhesive contacts. The altered cellular localisation of Rac in DX-treated cells would support this. HEF1/Cas-L (Human Enhancer of Filamentation/cas-like) was isolated in a screen for human proteins that confer morphoregulatory changes leading to filamentous budding in yeast, and independently due to its homology with p130cas (Law *et al.*, 1996; Minegishi *et al.*, 1996). Analysis of macrophage lysates revealed no change in expression of HEF1 between untreated and DX-treated cells (results not shown). Interestingly, we were only able to detect the 55kDa form of the protein that arises from cleavage of the full length 105kDa protein at G2-M (Law *et al.*, 1998). As macrophages are terminally differentiated cells, the significance of the possible presence of mitotic proteins is not known. Expression of the third member of the p130cas family of adaptor proteins, Efs/Sin (embryonal Fyn substrate/src interacting) was not assessed as only the murine form has so far been cloned (Ishino *et al.*, 1995). Homologues of Crk, DOCK180, and Rac are present in the nematode worm, (CED-

2, -5, and -10), however as yet no homologue of p130cas has been identified, despite cloning the entire *C.elegans* genome. *C.elegans* encodes a potential adaptor protein CED-6, which contains phosphotyrosine binding domains, however the gene encoding CED-6 does not belong to the same complementation group of phagocytosis mutations as *ced-2, -5, -10* and *–12*, and is thought to interact with the intracellular domain of CED-1, a putative membrane receptor. Mutations in both gene groups are required for full inhibition of engulfment (Ellis *et al.*, 1991). It is therefore possible that CED-6 may interact with CED-2/CED-5/CED-10 in the worm or the human homologue with Crk/DOCK180/Rac or the newly identified ELMO in mammalian cells. Such interaction may compensate for p130cas loss in DX-treated macrophages. As no mAb for CED-6 was available, we looked for mRNA expression of *ced-6* and *ced-7* by RT-PCR. In preliminary experiments both transcripts appeared to be present in untreated and DX-treated monocyte/macrophages (results not shown).

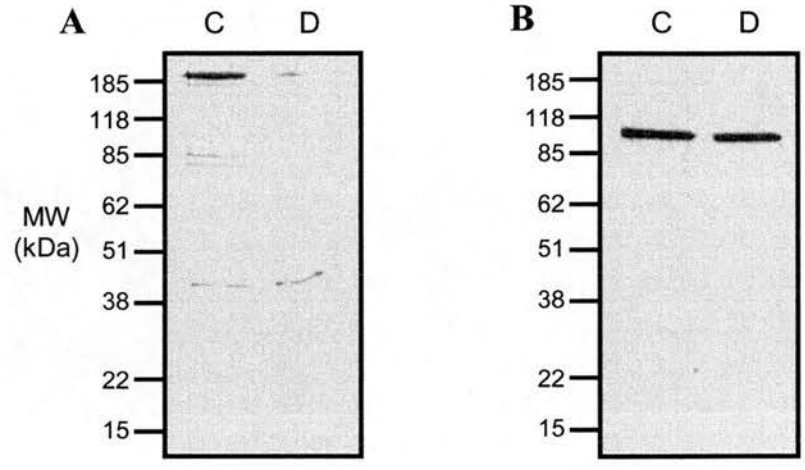


Figure 10. Altered Rac GEF expression in DX treated macrophages
Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days. Macrophage cell lysates were assessed for expression of **A** Tiam 1 (202 kDa), and **B** Vav (95kDa) by SDS-PAGE and Western blotting. Lanes labelled (C) are from untreated control macrophages and (D) are from DX-treated macrophages. Antibody dilutions Tiam1, 1:250; Vav, 1:1000.

Discussion

Glucocorticoids alter macrophage morphology

The previous chapter documented how exposure of monocytes to glucocorticoids during the early phase of monocyte-macrophage differentiation resulted in a highly phagocytic macrophage phenotype. Here we demonstrate that the increase in phagocytosis is accompanied by a profound alteration of monocyte/macrophage morphology. No morphological changes were apparent if monocyte/macrophages were treated with glucocorticoids late during differentiation. DX treatment after 4 days maturation in the absence of glucocorticoids did not alter cell morphology, and only resulted in a modest increase in phagocytosis. This suggests that DX does not alter cytoskeletal organisation directly, but induces morphological changes via induction of regulatory genes. Glucocorticoids have been reported to induce and stabilise actin polymerisation in human endometrial cells via a rapid non-genomic action involving the down regulation of cellular cAMP (Koukouritaki *et al.*, 1997; Koukouritaki *et al.*, 1996), and paxillin (Koukouritaki *et al.*, 1999). Additionally, Luini and colleagues demonstrated the remodelling of the actin cytoskeleton and formation of stress fibres in a number of cell types due to an enhancement of caldesmon expression, a ubiquitous actin- and calmodulin binding protein which synergizes with tropomyosin to stabilise microfilaments (Castellino *et al.*, 1992; Castellino *et al.*, 1995). We have not investigated a role for caldesmon in our system as we do not observe stress fibre formation in either DX-treated or untreated monocyte/macrophages. Furthermore, the highly phagocytic nature of DX-treated cells would require a dynamic regulation of cytoskeletal structure suggesting a decrease in actin filament stabilisation. Visualisation of cytoskeletal components with immunofluorescent staining demonstrated altered actin organisation in DX-treated monocyte/macrophages with localisation around the periphery of the cell similar to actin arrangement in p130cas -/- fibroblasts (Honda *et al.*, 1998). In addition to the downregulation of p130cas expression, inhibition of Pyk2 and paxillin phosphorylation is likely to contribute to the "rounded" morphology of DX-treated macrophages. Inhibitors of Pyk2 phosphorylation abrogate monocyte cell spreading

and motility (Watson *et al.*, 2001), and fibroblasts which lack Pyk2 expression demonstrate a more rounded phenotype (Sieg *et al.*, 1998).

Paxillin interacts with a number of proteins able to regulate cytoskeletal organisation (for review see Turner, 2000). Paxillin contains a number of protein-protein interaction domains including alpha-helical LD motifs, zinc finger containing LIM (Lin-11, Isl-1, Mec-3) domains, a proline rich region, plus binding motifs created by phosphorylation of tyrosine or serine residues. Decrease in paxillin phosphorylation may disrupt paxillin/Crk interactions in addition to other signalling mediators pertaining to cytoskeletal organisation. Interestingly, mutations in PP2A a serine phosphatase, increase serine phosphorylation of paxillin and promote cell spreading (Ito *et al.*, 2000). Assessment of serine phosphorylation of paxillin and its possible modulation by glucocorticoids was not undertaken in this study but presents an attractive target for future investigation. Paxillin has recently been shown to interact directly with the α_4 integrin tail, which inhibits cell spreading and reduces the formation of focal adhesions in Jurkat T cells and mouse embryonic fibroblasts (Liu *et al.*, 1999). α_4 integrins are largely restricted to mononuclear leukocyte lineages (Giancotti, 2000) and may therefore be a cell-specific candidate for paxillin regulation in macrophages. It would be an intriguing possibility if paxillin/α_4 interactions were independent of paxillin tyrosine phosphorylation, and may therefore be potentiated in DX-treated cells. Two homologues of paxillin have been identified in mammalian cells, Hic-5 (Matsuya *et al.*, 1998; Thomas *et al.*, 1999) and leupaxin (Lipsky *et al.*, 1998). Both are also able to bind α_4 integrins, Pyk2 and FAK (Hic-5 only) *in vitro*. Recent work described Hic-5 as a negative regulator of integrin mediated cell spreading. Overexpression of Hic-5 in NIH 3T3 cells sequestered FAK from paxillin, decreased paxillin phosphorylation, and prevented interaction of paxillin and Crk (Nishiya *et al.*, 2001). Src activity was also decreased in Hic-5 overexpressing cells suggestive that expression of this paxillin homologue may alter adhesion signalling by preventing phosphorylation of components such as p130cas by Src family kinases. Changes induced by Hic-5 overexpression were dependent on the presence of FAK as decreased paxillin phosphorylation was not apparent in FAK -/- fibroblasts. We do not detect FAK expression in our monocyte-

derived macrophages, however Pyk2 has been shown to compensate for certain FAK functions when overexpressed in FAK -/- cells (Klingbeil *et al.*, 2001). Increased Hic-5 (and possibly leupaxin) expression after DX-treatment could therefore promote a reduction in paxillin phosphorylation by competition for Pyk2 binding resulting in increased cell rounding characteristic of DX-treated macrophage morphology. These possibilities could be tested when specific reagents are available.

Changes in kinase/phosphatase activity after DX-reprogramming

Src and src-family kinases mediate phosphorylation of cytoskeletal components in response to integrin ligation. Src has been show to directly phosphorylate and activate Pyk2 in addition to p130cas in fibroblasts (Sakai *et al.*, 1994; Sieg *et al.*, 1998). In monocytes and macrophages, the major src-family kinases expressed are $p59/61^{hck}$ (Hck), $p58^{c\text{-}fgr}$ (Fgr), and $p53/56^{lyn}$ (Lyn) (Tsygankov and Bolen, 1993). Peritoneal exudate macrophages from *hck-/-fgr-/-* mice showed reduced phosphorylation of a number of cytoskeletal associated proteins, including paxillin and Pyk2, and a dysregulation of paxillin and actin localisation on adhesion to fibronectin (Suen *et al.*, 1999). Additionally p130cas promotes Hck autophosphorylation and activation in an *in vitro* cell free assay system (Pellicena and Miller, 2001). Hck and Fgr may therefore be targets for DX modulation directly by changes in expression or indirectly through reduced activation in the absence of p130cas.

Decreased phosphorylation of paxillin and Pyk2 may be mediated by an increased activity/expression of specific phosphatases. SHP-1 (SHPTP1) dephosphorylates Pyk2 (Kumar *et al.*, 1999), and is expressed primarily by haematopoietic cells. Mutation of murine SHP-1 results in the "motheaten" phenotype, characterised by accumulation of macrophages and neutrophils in the lung (Shultz *et al.*, 1984), and increased $\alpha_M\beta_2$ integrin-mediated macrophage cell adherence and spreading (Roach *et al.*, 1998). However, examination of macrophage lysates for SHP-1 expression revealed an actual decrease in expression after DX treatment (data not shown). Another possible candidate for DX modulation is PTP-PEST. PTP-PEST inhibits phosphorylation of Pyk2, p130cas and paxillin (Davidson and Veillette, 2001; Lyons

et al., 2001; Shen *et al.*, 1998). Analysis of PTP-PEST activity after DX treatment may present a possible mechanism for the altered phosphorylation of adhesion signalling proteins observed.

Podosome formation and DX-reprogramming

In addition to regulating cell spreading and morphology, Pyk2, paxillin and p130cas have also been shown to regulate the formation of podosomes, specialised adhesion structures, in response to ligation of $\alpha_M\beta_2$, $\alpha_V\beta_3$, and β_1-integrins (Duong and Rodan, 2000; Nakamura *et al.*, 1998). Osteoclasts from p130cas -/- mice show deficiencies in actin ring formation and podosome assembly (Nakamura *et al.*, 1998). We would predict that the dysregulation of podosome formation by changes in p130cas expression, or Pyk2 and paxillin phosphorylation would affect cellular localisation and changes in activation of the downstream effectors of actin organisation, principally Rho family GTPases, and the Wiskott-Aldrich Syndrome protein (WASp) family. RhoA, Rac, and cdc42 are required for podosome formation in osteoclasts (Zhang *et al.*, 1995) and MNGCs (Ory *et al.*, 2000), and dendritic cells (Burns *et al.*, 2001). Changes in of Rho or Rac activity induce the dissociation and turnover of podosome structures (Ory *et al.*, 2000), and WASp deficient macrophages fail to form podosomes, exhibit compromised motility and show defective phagocytosis of apoptotic cells. This demonstrates a direct link between Rho family GTPases, actin organisation, and phagocytosis. Whether WASp functions in a similar signalling pathway during phagocytosis as adhesion is not yet known. Analysis of protein levels in macrophage lysates revealed that WASp is expressed at equivalent levels in both untreated and DX-treated cells (data not shown), therefore glucocorticoid programmed monocyte/macrophages may provide a useful model to study WASp function where expression permits phagocytosis but not podosome formation.

Adhesion and phagocytosis, two opposing functions?

Considering adhesion signalling can modulate macrophage phagocytosis, and many of the components of adhesive contacts are also involved in engulfment, could the

absence of podosomes facilitate phagocytosis in DX treated macrophages? MNGCs present only in untreated cell populations contain numerous podosomes and other adhesion structures (for detailed analysis of the effect of DX programming on giant cell formation see chapter 7). In our phagocytosis assay these cells are poorly phagocytic. Phenotypic analysis showed no alteration in the number of cells expressing cell surface receptors implicated in uptake of apoptotic particles in DX-treated vs. untreated monocyte/macrophages (where the proportion of MNGCs is higher) (see chapter 1). Low phagocytic potential may therefore be reflected in the limiting availability of membrane or cytoskeletal components in highly spread cells and is reflected in time-lapse video microscopy of macrophage cultures. MNGCs and large spread monocyte/macrophages showed ruffling membranes, but little extension of membrane processes. In contrast DX-treated cells were very active in extending cellular processes and capturing apoptotic cells.

In order to test this hypothesis we would be required to induce podosome formation in DX-treated cells. Replating DX-treated cells on "virgin" plastic induced strong adhesion as quantified by paxillin phosphorylation. Microscopic analysis has not yet been undertaken to assess whether this correlates with podosome formation, or if replating inhibits phagocytic capacity, but provides a potential model for such an analysis. As phosphorylation of paxillin can be induced in DX-treated macrophages the absence of phospho-paxillin in resting DX-cells may be due to a rapid turnover of weak, transient adhesion structures, where only a small percentage of available cytoskeletal components are involved. Such components including paxillin and actin could then be readily mobilised to areas of membrane in contact with an apoptotic cell without the need for *de novo* synthesis, or a dismantling of existing cytoskeletal structures. We would therefore predict an induction of phospho-paxillin after engagement of apoptotic particles. Technical difficulties precluded us investigating this directly. The average phagocytic signal in DX treated cultures is approximately 40% which is likely to be sufficient to detect changes after particle engagement. However phosphorylation may be transient and as phagocytosis is not synchronised during the assay the actual percentage of cells engulfing particles and consequently the paxillin phosphorylation signal at any one time may be far lower. The

contribution of paxillin present in the apoptotic neutrophil may also mask detection of phosphorylation. Assessment of the time course of paxillin phosphorylation after replating in conjunction with phagocytic capacity would present further evidence linking or divorcing adhesion and paxillin phosphorylation status with phagocytic capability. A time course may also enable assessment of the role of matrix laid down by monocyte/macrophages during differentiation on the phenotype and phagocytic capacity induced by DX. If DX programmed morphology was independent of matrix ligation of cell surface receptors, we would expect a recovery of the original phenotype shortly after initial adherence. Alternatively if the DX treated macrophage phenotype were influenced by the matrix laid down during culture, we would not expect recovery for a much longer period. Potential differences in matrix production after DX treatment raises further questions as to the effect of glucocorticoids during wound healing and fibrotic repair.

DX promotes cytoskeletal rearrangement

Increases in phagocytic capability conferred by glucocorticoids was not confined to the uptake of apoptotic cells. However the phagocytic advantage conferred by DX diminished with decreasing particle size. DX promoted the uptake of IgG-opsonized erthyrocytes, but showed little advantage for the uptake of either boiled zymosan or complement opsonized zymosan. This suggests that under conditions where less cytoskeletal and membrane organisation was required to physically engulf a particle, untreated cells would be able to perform with similar efficiency as DX treated macrophages. Furthermore blockade of PI3 kinase activity did not produce such a profound inhibition of opsonized erythrocyte phagocytosis when compared with apoptotic cell uptake in DX treated cells and did not alter uptake of whole zymosan or complement opsonized zymosan. As different receptor mechanisms are employed for the uptake of each particle a direct comparison between different phagocytosis pathways is difficult, however preliminary evidence suggests that DX does not alter FcR or complement receptor expression. Assessment of phagocytosis of different sized latex beads coated with phosphatidylserine, IgG or complement proteins could directly determine whether the phagocytic advantage of DX treated, compared to

untreated cells, is dependent on cytoskeletal plasticity for each of the three phagocytosis mechanisms and not the receptor engaged. Alternatively direct comparison of engulfment of differentially opsonized apoptotic cells would determine if increased phagocytic potential of DX-treated cells were receptor dependent. Augmentation of phagocytic capacity for both IgG-opsonized and apoptotic particles provides further evidence that apoptotic cell uptake is mediated via a "modified zippering mechanism". In a similar fashion to FcR-mediated phagocytosis, a number of cell surface receptors may be required for successful engulfment of apoptotic bodies. This may consist of any number of phagocytic receptors so far described. Failure to completely abrogate uptake via blockade of a single receptor may therefore not be due to functional redundancy but by a physical/spatial redundancy.

DX augments Rac activity

Increases in phagocytic activity after DX "programming" correlated with heightened efficiency for the rearrangement of cytoskeletal components required for physical uptake of particles. In mammalian systems, work modelling dendritic cell uptake of apoptotic particles using human epithelial kidney 293T cells transfected with $\alpha_v\beta_5$ integrins demonstrated that uptake via Crk/DOCK180/Rac was associated with p130cas phosphorylation. Additionally Ravichandran and colleagues demonstrated that overexpression of Crk or DOCK180 in CHO cells promoted phagocytosis (Tosello-Trampont *et al.*, 2001). We would predict that loss of p130cas expression would disrupt formation of the Crk/DOCK180 complex, therefore the high levels of Rac activity in DX-treated cells presents us with a paradox. Sister proteins such as HEF-1 or Efs/Sin may compensate in the absence of p130cas. HEF-1 has been shown to interact with Crk (Kanda *et al.*, 1997; Law *et al.*, 1996; Manie *et al.*, 1997; Minegishi *et al.*, 1996). Changes in localisation patterns of alternate binding partners may concentrate active Rac to areas of the membrane participating in engulfing particles for example lipid rafts (Tosello-Trampont *et al.*, 2001), and may account for the altered localisation of whole Rac in DX-treated macrophages. Alternatively glucocorticoids may augment Rac activity by increased expression of Rac specific

GEFs. Currently 8 Rac specific GEFs have been identified (for review see Scita *et al.*, 2000). We examined the expression of two at the protein level. DX down regulated the expression of Tiam1, and prevented Vav phosphorylation (which is required for activation of Rac), suggesting that these proteins were not involved in the increase of Rac activity with DX-treatment. A number of other Rac specific GEFs remain to be examined in our system including mediators of Rac activation downstream of Ras and Cdc42. Ras has been shown to activate Rac via PI-3 kinase and the GEFs Sos-1 or Vav (Han *et al.*, 1998; Ridley *et al.*, 1992; Walsh and Bar-Sagi, 2001). As Ras activity is not required for the uptake of apoptotic cells or opsonized particles, DX-treated macrophages may employ a Ras independent PI-3 kinase-Rac pathway (Nobes *et al.*, 1995). Cdc42 is required for phagocytosis of apoptotic cells and IgG-opsonized particles. One of the mechanisms whereby Cdc42 is thought to mediate activation of Rac is via PAK (also a target for activated Rac), which forms a complex with PIX (PAK Interacting Exchange Factor), a Cdc42/Rac GEF. Interestingly paxillin can localise PIX (and possibly therefore Rac activity) at focal adhesions through an ADP-ribosylation factor GTPase-activating protein (ARF-GAP) called PKL (Turner, 2000). This interaction is via the LD4 domain of paxillin and therefore independent of tyrosine phosphorylation. Changes in paxillin phosphorylation with DX-treatment may promote interaction with alternate binding partners, and in addition to altered paxillin cellular localisation, provide a mechanism whereby Rac activity is recruited away from sites of focal contact to areas of membrane engaging apoptotic particles. We were unable to directly show a role for Cdc42 in our DX-treated macrophage phenotype. Although reagents used for detection of Rac activity also allow identification of active Cdc42, technical difficulties prevented us observing GTP-bound Cdc42 in either untreated or DX-treated cells.

Work examining the role of the p130cas/Crk complex and Rho GTPases in *Yersinia spp.* infection may provide a useful tool for the further extrapolation of Rho family signalling during phagocytosis. *Yersinia* are taken up by epithelial cells via interaction of the bacteria protein invasin with β_1 integrins (Fallman *et al.*, 1995; Isberg and Leong, 1990). This induces phosphorylation of p130cas, Crk binding,

Rac activation and engulfment of bacteria (Weidow *et al.*, 2000). *Yersinia* however, inhibit this same machinery to avoid immune detection by myeloid cells via the secretion of Yops (*Yersinia* outer proteins) (Cornelis and Wolf-Watz, 1997). YopH is a phosphatase which targets p130cas phosphorylation, inhibiting p130cas/Crk coupling and Rac activation (Black and Bliska, 1997; Hamid *et al.*, 1999; Persson *et al.*, 1997; Weidow *et al.*, 2000). Additionally YopE has been recently characterised as a Rac specific GAP (Black and Bliska, 2000; Von Pawel-Rammingen *et al.*, 2000). Analysis of human umbilical vein endothelial cell cultures infected with a *Yersinia* strain that secreted only YopE demonstrated that direct Rac activation was not inhibited, although Cdc42 mediated Rac activity was (Andor *et al.*, 2001). Examination of phagocytosis of apoptotic cells and basal levels of Rac activity in DX-treated monocyte/macrophages cultures infected with a *Yersinia* strain secreting only YopE would provide strong evidence for Cdc42 mediated Rac activation in our system. In a wider context, patients on long-term glucocorticoid treatment demonstrate increased susceptibility to bacterial infection. Glucocorticoids have been shown to increase uptake of *Staphylococcus aureus* (van der Goes *et al.*, 2000), however we would predict that downregulation of p130cas would mimic dephosphorylation by YopH and prevent *Yersinia* uptake. DX treatment may therefore be detrimental for functional immunity towards pathogens that employ similar mechanisms to avoid immune detection. Finally, considering the profound effects on gene expression seen during glucocorticoid "programming" of monocyte-macrophage differentiation observed so far, its is likely that DX may induce the expression of novel GEFs. Utilisation of current "chip" technology would allow the creation of custom arrays for analysis of untreated vs. DX-treated monocyte/macrophage mRNA expression, identifying novel glucocorticoid inducible genes.

The majority of studies assessing Rac activation, with the exception of the dendritic cell work, are performed in either non-phagocytosing or "non-professional phagocytes". These cells are very likely to require p130cas for Rac activation in the context of adhesion but these cells never or rarely have a requirement for Rac during phagocytosis. In such studies functional readout for Rac activation is the assessment

of cell spreading or membrane ruffling. Few investigations measure Rac activity directly or localise active Rac within the cell. The use of dominant negative or constitutively active Rac constructs are very useful tools but may not be subtle enough to activate or inhibit individual Rac functions. Work by Albert *et al.* (2000) demonstrated p130cas phosphorylation after adherence of transfected 293 cells to vitronectin, and that adherence to vitronectin promoted phagocytosis in both dendritic cells and 293 cells. If adherence induces p130cas phosphorylation, promoting Rac activity in an adhesion context, high levels of active Rac in a non-phagocytosing cell transfected with a "phagocytic receptor" may promote phagocytosis by default. In a similar fashion expression of constitutively active Rac would promote uptake. However these mechanisms are unlikely to be as efficient as in macrophages and may account for the length of assay required to see significant levels of phagocytosis (2-4 hr as opposed to our 20-min assay). Interestingly, worms with defects in the two nematode integrin molecules pat-2 and ina-1 do not show defects in corpse clearance (Gumienny *et al.*, 2001), although CED-2, -5 and –10 are required. *Ced*-6 encodes an adaptor protein with phosphotyrosine binding motifs, a proline rich region at its C terminus and a possible pTyr motif. Genetically, *ced-6* is not in the same complementation group as *ced-2, -5*, and *–10*, and is therefore unlikely to fulfil a "p130cas-like" role in the worm. Preliminary experiments demonstrated the presence of mRNA transcripts for the human homologues of *ced-6* and *–7* in both DX treated and untreated macrophages (results not shown). Furthermore we were able to inhibit phagocytosis of apoptotic neutrophils by DX-treated and untreated macrophages by 50-90% with the ABC1 (CED-7) antagonist, glyburide (see chapter 3), suggesting this pathway is functional. Whether human CED-6 can bind Crk and promote Rac activation in mammalian cells remains to be determined. Further characterisation of the function of CED-12/ELMO another putative adaptor protein may allow further elucidation of the pathways involved in mediating recognition of the apoptotic target which may be mediated by CED-1, -6, -7 and engulfment requiring CED-2, -5, and 10. Expression and function of ELMO in DX-treated macrophages presents a very exciting target for future work.

In higher organisms, phagocytosis of apoptotic cells is likely to be highly regulated with expression of promoters of phagocytosis, in addition to proteins that may prevent phagocytosis of apoptotic cells where it would be detrimental to the organism, for example dendritic cell presentation of self-antigens from phagocytosed apoptotic cells (Albert *et al.*, 1998a; Albert *et al.*, 1998b; Inaba *et al.*, 1998). Immature dendritic cells have heightened capacity for the uptake of apoptotic cells and are able to cross-present antigens from apoptotic cells to cytotoxic T lymphocytes (Albert *et al.*, 1998a; Albert *et al.*, 1998b). In addition, dendritic cells have also been shown to process antigen and present epitopes in MHC class II from necrotic and apoptotic cells. (Inaba *et al.*, 1998). However as immature dendritic cells have low levels of co-stimulatory molecules, cross-priming and MHCII presentation results in tolerance. In contrast, mature dendritic cells which have increased expression of co-stimulatory molecules have decreased capacity for the uptake of apoptotic cells. This is thought to be due to a down regulation of phagocytic receptors, $\alpha_V\beta_5$ (Albert *et al.*, 1998a). Uptake of apoptotic cells by immature dendritic cells in the presence of an inflammatory stimulus, which would induce co-stimulatory activity, has the potential to successfully activate cytotoxic T lymphocytes to self-antigen, resulting in the death of the APC. Macrophages have the potential to express co-stimulatory molecules in response to inflammatory stimuli, if this is married with a continued phagocytic capacity, activation of self-responses may occur. Albert *et al.*, found that macrophages were unable to cross-present, and inclusion of macrophages into dendritic cell cultures containing apoptotic cells actually decreased the cross-presenting ability of dendritic cells (Albert *et al.*, 1998a). However this does not exclude the potential for macrophages to present self-antigens on MHC class II. It is proposed that whereas dendritic cells phagocytose apoptotic cells via $\alpha_V\beta_5$ which engages intracellular processing pathways leading to presentation, macrophages employ $\alpha_V\beta_3$ for uptake resulting in the degradation of the apoptotic material (Albert *et al.*, 2000). Albert and colleagues have proposed a requirement for p130cas during the uptake of apoptotic cells by dendritic cells, however our data unequivocally demonstrates no requirement for p130cas during macrophage phagocytosis, furthermore its absence may promote clearance of corpses. It would be interesting to speculate that p130cas may function

as a cellular switch governing the intracellular pathways with which ingested apoptotic material is processed. Recent work describes the horizontal transfer of oncogenes via uptake of apoptotic tumour cells by fibroblasts (Bergsmedh *et al.*, 2001). This would presumably require engagement of a third specialised processing pathway for apoptotic cells distinct from the degradative or antigen presenting paths currently described. How DX-reprogramming affects the fate of proteins from phagocytosed particles, and the subsequent effects on macrophage function, for example antigen presentation or release of anti-inflammatory mediators (Fadok *et al.*, 1998) must be addressed in order to further understand the potential wide ranging effects of glucocorticoid therapy.

We could propose a dual role for p130cas in which it is an important mediator of Rac activation in adhesion, but a negative regulator of phagocytosis. Which pathway mediated would be dependent on upstream signalling. For example Tiam-1, a potent Rac-GEF, poorly induces Jun N-terminal kinase (JNK) activity but strongly upregulates Pak1 (Zhou *et al.*, 1998). Function of both kinases requires Rac activity therefore the upstream signalling that regulates Rac may also determine the final cellular response. Phosphorylation of p130cas after integrin engagement by macrophage adhesion to fibronectin or dendritic cell engagement of apoptotic cells may act to transiently sequester p130cas, mimicking the loss of p130cas expression in DX treated cells, enabling activated Rac to be directed towards areas of membrane engaging in phagocytosis. In order to determine whether p130cas does play such a pivotal role we sought to re-express the protein in DX-treated macrophages and determine subsequent phagocytic capability and morphological changes. These studies will be described in detail in chapter 6.

CHAPTER 5: THE EFFECT OF GLUCOCORTICOIDS ON MAPK SIGNALLING

Introduction

Cell migration is a fundamental process required for growth and development, wound repair, angiogenesis and the inflammatory response. Dysregulation of cell motility promotes tumour invasion, metastasis, and underlies the pathogenesis of developmental and immunodeficiency disorders (Keely *et al.*, 1998; Lauffenburger and Horwitz, 1996; Raff, 1992; Steller, 1995). Directed migration requires the regulation of four cellular activities: polarization towards chemotactic stimuli, extension of membrane processes, cell contraction, and formation and turnover of adhesive contacts. Stimuli from chemokines, or cell-ECM interactions are co-ordinated principally by the Rho family of GTPases and their down stream effectors which mediate organisation of the actin cytoskeleton (Keely *et al.*, 1998; Keely *et al.*, 1997; Tapon and Hall, 1997) (for review see Jones *et al.*, 1998). Initially, polarization of chemokine receptors was thought to promote directed movement, however it has been subsequently shown that receptors are distributed diffusely around the cell (van Es and Devreotes, 1999), and accumulation of lipid products of PI 3-kinase at the leading edge of the cells produces a steep internal gradient to promote cell polarity and chemotaxis (Hirsch *et al.*, 2000; Li *et al.*, 2000; Sasaki *et al.*, 2000; Servant *et al.*, 2000). Inhibitors of PI 3-kinase, for example LY294002, reduce chemoattractant motility in a number of leukocytes: neutrophils (Knall *et al.*, 1997; Niggli and Keller, 1997), T cells (Vicente-Manzanares *et al.*, 1999) natural killer cells (al-Aoukaty *et al.*, 1999) and macrophages (Vanhaesebroeck *et al.*, 1999). As the Rho-family GTPases, Rac1 and Cdc42 can be regulated by PI 3-kinase lipid products in leukocytes (Akasaki *et al.*, 1999), and their function is required for chemotactic movement, it is likely that GTPase function translates the internal gradient of lipid mediators into directionality. Cdc42 function is not required for random migration, but is essential for macrophage movement towards CSF-1 (Allen *et al.*,

1998), and neutrophils from Rac2 deficient mice cannot chemotax towards fMLP (Ambruso *et al.*, 2000; Roberts *et al.*, 1999). Lipid products are thought to activate a cellular tyrosine kinase, which phosphorylates Rho-family specific GEFs. One candidate is Vav, a substrate for the tyrosine kinases Lck and Syk (Gulbins *et al.*, 1993; Han *et al.*, 1997). GEF activity is increased on tyrosine phosphorylation, and the presence of the lipid products of PI 3-kinase (Han *et al.*, 1998). Macrophage movement is initiated by the extension of filopodia and lamellipodia regulated by Cdc42 and Rac respectively (Kozma *et al.*, 1995; Nobes and Hall, 1995; Ridley and Hall, 1992). During extension of processes adhesive contacts and podosomes are formed under the control of Cdc42/WASp/Arp2/3 complexes, and function to stabilise membrane structures and maintain cell polarity (Burns *et al.*, 2001; Linder *et al.*, 1999; Zicha *et al.*, 1998). Podosomes may be important sites of cross talk between cytoskeletal and adhesive contacts during the regulation of directed migration. Interestingly another effector of cdc42, PAK, has also been shown to be defective in WAS patients (Haddad *et al.*, 2001). Contraction of cytoplasmic actomyosin filaments assembled in response to myosin light chain (MLC) phosphorylation (Adelstein, 1983; Burridge and Chrzanowska-Wodnicka, 1996; Yoshioka *et al.*, 1998), plus the release of contact sites in the trailing edge of the cell, mediated by RhoA (Alblas *et al.*, 2001; Worthylake *et al.*, 2001) induces forward movement. Extracellular regulated kinase (ERK) can regulate MLC-kinase, promoting phosphorylation of MLC, and assembly of myosin fibrils (Klemke *et al.*, 1997). Phosphorylation of MLC can also be augmented directly by Rho-Kinase (a Rho GTPases effector) (Amano *et al.*, 1996) or indirectly by phosphorylation and inactivation of myosin phosphatase (Kimura *et al.*, 1996).

The mechanisms modulating random movement or persistent directional movement in the absence of a chemokine gradient have not been so extensively studied. WASp and Cdc42 are not required for random movement (Linder *et al.*, 2000; Zicha *et al.*, 1998), however inhibition of RhoA or Rac does perturb haptotactic movement (Jones *et al.*, 1998). Additionally the adapter protein Shc which can mediate src kinase activation of

Ras signalling pathways has been implicated in the regulation of random migration in carcinoma cells (Collins *et al.*, 1999; Gu *et al.*, 1999).

In addition to Rho family GTPases and PI 3-kinase, multiple signals have been shown to regulate migration, PLCγ (Chen *et al.*, 1994a), FAK (Ilic *et al.*, 1995), c-src and family kinases (Fincham and Frame, 1998), C3G (Uemura and Griffin, 1999), Pyk2 (Watson et al., 2001; Williams and Ridley, 2000), Tiam1 (Michiels *et al.*, 1995; Sander *et al.*, 1998) and MAPK (Anand-Apte *et al.*, 1997; Klemke *et al.*, 1997). Functional studies have described how different regulators modulate distinct aspects of migration in different cell types. For example Klemke and colleagues demonstrated that during fibroblast migration p130cas/crk coupling and Rac activation were required for membrane ruffling whereas, ERK was required for cell contractility (Cheresh *et al.*, 1999). In addition to their role in cell migration Mitogen Activated Protein Kinase (MAPK) cascades are involved in a number of cellular programs including cell differentiation, cell division, and cell death (for reviews see Ip and Davis, 1998; Lewis *et al.*, 1998; Nebreda and Porras, 2000). Induction can be triggered by ECM-integrin engagement, or occupancy of cytokine or growth factor receptors. The MAPK cascade is composed of five branches or modules: the extracellular-regulated kinase 1 and 2 (ERK1/2) cascade which regulates growth and differentiation, and more recently has been shown to be an important regulator of cell migration and survival (Cho and Klemke, 2000); c-Jun N-terminal kinase (JNK) and p38 MAPK cascades which are activated in response to stress, for example during inflammation and apoptosis; plus ERK3 and ERK5 cascades thought also to modulate proliferation (Cheng *et al.*, 1996; English *et al.*, 1999; Robbins *et al.*, 1993; Zhou *et al.*, 1995). Activation of the terminal kinases, in this case ERK1 and 2, occurs after sequential phosphorylation (and activation) of components of a four step cascade. This commences on activation of a G-protein, typically Ras, which activates a MAPKKK (Raf amongst others), followed by activation of a MAPKK (MEK) before induction of MAPK (ERK) activity. The cascade enables not only signal amplification but also cross-talk from stimuli which may modulate the duration and amplitude of the response, and consequently the biological outcome (Schaeffer and

Weber, 1999). DX-treated monocyte/macrophages show a heightened capacity for reorganisation of cytoskeletal components, and an augmented Rac activity. However DX induces a downregulation of p130cas expression and other signalling intermediates which have been implicated in migration, Tiam 1, C3G, plus a decrease in Pyk2 phosphorylation. We also observe a loss of podosome formation after DX-treatment. Little work has explored how cell migration contributes to phagocytosis of apoptotic cells, however we would predict that efficient clearance of apoptotic cells is likely to be highly dependent on cell motility *in vivo*. Furthermore cytoskeletal plasticity accompanying a migratory phenotype would promote the functional rearrangement of intracellular components required for phagocytosis. In order to investigate the effect of DX programming on macrophage migration we assessed ERK function, and observed macrophage migration *in vitro*.

Results

Glucocorticoids downregulate basal ERK activity

To assess if DX altered basal activity of ERK 1 and 2 we investigated whether ERK was phosphorylated by western blotting of macrophage cell lysates. Monocyte/macrophages were matured for 5-days in media containing autologous serum, plus or minus 1μM DX. Cells were lysed in either an NP-40 based lysis buffer or RIPA buffer, whilst adherent to tissue culture plastic, to prevent any changes in ERK activity during cell detachment. Whole cell lysates were separated by SDS-PAGE and analysed by western blotting, firstly with mAbs recognising total ERK2 to ensure equal loading, and secondly with a mAb corresponding to the active enzyme. This recognises an epitope created by phosphorylation of a Thr-Glu-Tyr motif in the activation loop of ERK1 and 2. ERK2 was expressed at equivalent levels in untreated and DX-treated cells. Due to cross-reactivity of the antibody, we were also able to visualise a 44kDa band corresponding to ERK1, which also showed no change in expression. Assessment of ERK phosphorylation revealed high levels of basal enzyme activity in untreated macrophages,

which was significantly inhibited in DX-treated monocyte/macrophages (fig. 1). ERK activity can be stimulated by serum factors, for example lysophosphatidic acid (Dikic *et al.*, 1996), and serum starvation of cell lines reduces basal ERK activity. To ensure that downregulation of ERK activity in DX-treated cells was not due to a premature depletion of serum factors by increased metabolism or proteolytic activity in the glucocorticoid treated cultures, we "fed" macrophages with media containing fresh autologous serum 24hrs prior to analysis of ERK activity. Despite serum replenishment, levels of ERK activity remained constant in untreated macrophages, with a similar downregulation in DX-treated cells (results not shown). This suggested that inhibition of ERK activity might represent a component of DX-programming of monocyte-macrophage differentiation in concert with the morphological changes and increase in phagocytosis observed. To examine whether changes in ERK activity followed a similar pattern to observed morphological changes described in chapter 4, we cultured monocytes/macrophages for up to 5 days in the presence or absence of 1μM DX as previously described. Lysates were made at 24hr intervals from time 0 to 5 days. Additionally cells were cultured in the absence of DX for 4 days, then treated with DX for 24 hrs prior to analysis. The time course of monocyte maturation revealed that ERK activity was reduced after 24 hrs of culture, which might be interpreted as a direct effect of glucocorticoids on kinase function. However, dexamethasone treatment after cells had matured in culture for 4 days without glucocorticoid showed no changes in ERK activity, demonstrating that downregulation of basal ERK function was part of DX reprogramming of macrophage differentiation.

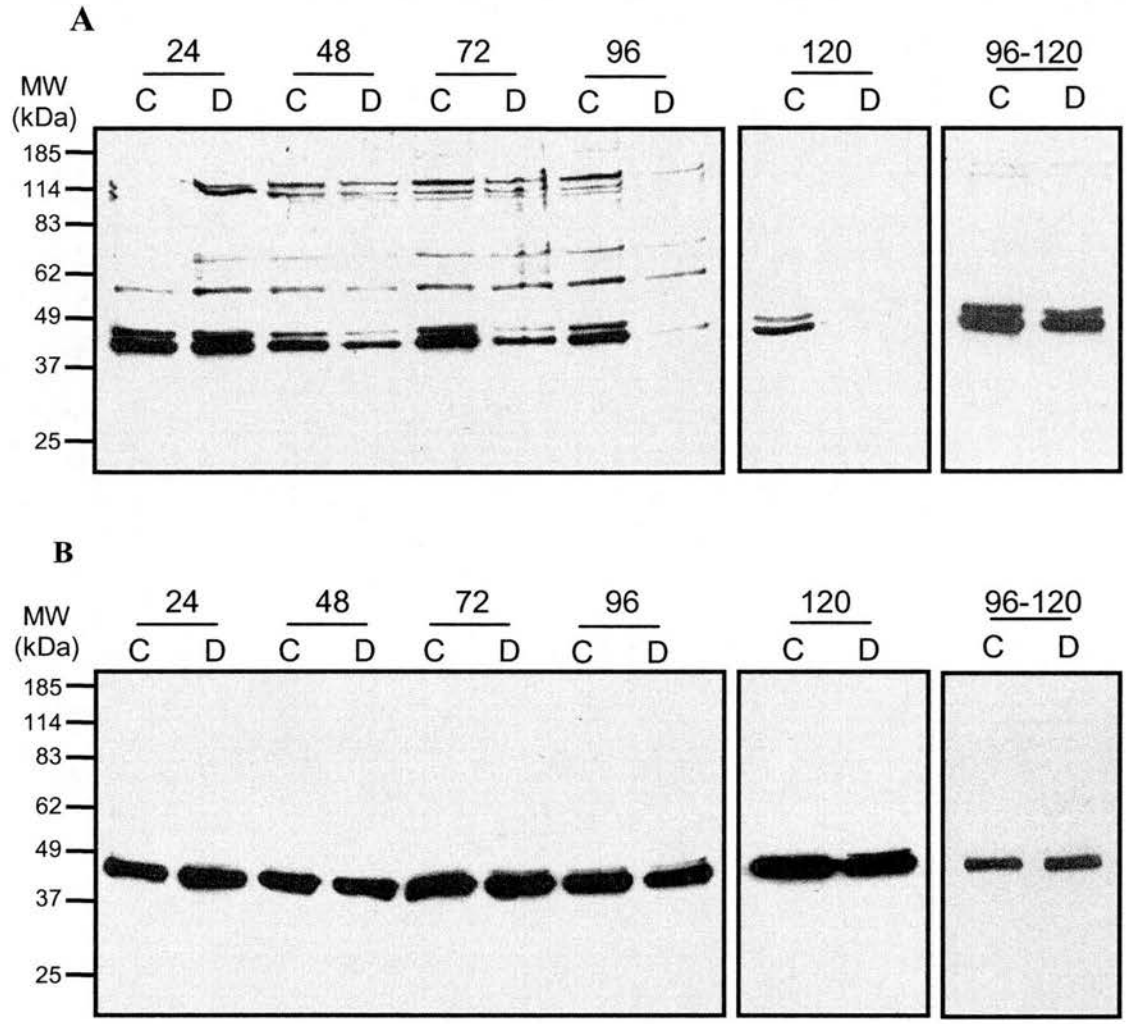


Figure 1. Effect of DX on ERK activity
Adherent peripheral blood monocytes were cultured for up to 5 days in the presence (D) or absence (C) of 1μm DX, for the periods shown (in hours). Macrophage cell lysates were assessed for expression of **A** phospho-ERK1/2 (active) (p44/42 kDa) and **B** total ERK2 (42 kDa) by SDS-PAGE and Western blotting. Antibody concentrations: pERK and ERK, 1:2000.

Down-regulation of ERK activity does not contribute to glucocorticoid augmented phagocytic capability

As a reduced ERK activity correlated with the acquisition of phagocytic capacity we therefore wished to test directly if ERK activity inhibited phagocytosis of apoptotic cells. Pre-treatment of 5-day monocyte/macrophages with 50nM of the pharmacological ERK inhibitor PD98059, for 30 min prevented ERK phosphorylation in non-DX-treated cultures, as assessed by western blotting of control macrophage lysates, see figure 3. Pre-incubation of monocyte/macrophages with PD98059 did not however alter levels of phagocytosis of apoptotic neutrophils by either non-DX-treated macrophages, or as a control for potential non-specific effects, DX-treated cells (fig. 2). Analysis was conducted using both the plate based and flow cytometric phagocytosis assay (see chapter 1).

Induction of ERK activity in DX-treated macrophages

ERK activity had no effect on phagocytosis in our *in vitro* assay system, however reduction in kinase function has implications for migration and phagocytosis *in vivo*. Cellular movement at the inflammatory site is likely to be required for the efficient clearance of apoptotic cells. Time lapse video microscopy of an *in vitro* phagocytosis assay showed little movement of either untreated or DX-treated monocyte/macrophages within a 20-minute assay period, although extension of cytoplasmic processes, sufficient for phagocytosis was readily observed (I. Dransfield, personal communication). We may not observe active movement during the phagocytosis assay, as our *in vitro* system is likely to lack specific matrix or chemotactic stimuli that would induce movement *in vivo*. To investigate if DX-treated monocyte/macrophages were able to activate ERK signalling cascades in response to classical inflammatory stimuli, we pre-incubated untreated and 5-day DX-treated monocyte/macrophages with either 1μg/ml LPS, 10ng/ml TNF, 1nM fMLP or 50nM PD98059 (as a control for the phagocytosis studies), for 30 minutes, prior to lysis and analysis of ERK phosphorylation by western blotting. Both LPS and TNF induced ERK phosphorylation in DX-treated macrophages, and LPS augmented existing activity in control untreated cells (fig. 3). Surprisingly, although

fMLP induces a calcium spike in monocyte/macrophages, demonstrating the presence of functional fMLP receptors (K. Ross personal communication), and has been shown to activate ERK in neutrophils (Avdi *et al.*, 1996; Chang and Wang, 1999; Nick *et al.*, 1997), the peptide failed to induce ERK activation in either untreated or DX-treated macrophages (fig. 3).

In addition to inflammatory mediators, MAPK signalling pathways can also be triggered by engagement of integrin receptors. Macrophages have the capacity to secrete ECM components *in vivo* during wound healing and tissue repair and during *in vitro* maturation and culture. Differences in matrix production between DX-treated and untreated monocyte/macrophages could influence the utilisation of a repertoire of adhesion receptors that fail to maintain basal ERK activity. "Replating" cells onto tissue culture plastic has been reported to induce a spike of MAP kinase activity in response to integrin engagement (Chen *et al.*, 1994b; Clark and Hynes, 1996; Miyamoto *et al.*, 1996; Morino *et al.*, 1995; Schlaepfer *et al.*, 1994). This may persist at 60-100% of maximal levels for periods ranging from 15 minutes to 2 hours or more, followed by either a gradual decline (Chen *et al.*, 1994b; Clark and Hynes, 1996) or a rapid decrease (Miyamoto *et al.*, 1996; Morino *et al.*, 1995). The kinetics of ERK activation will depend on the cell type and integrin-ligand interaction involved. As was hypothesised for paxillin phosphorylation in chapter 4, if the low ERK activity in DX-treated macrophages were due specifically to "reprogramming", replating would induce an initial kinase activation, which would diminish rapidly, possibility from engagement of adhesion receptors employed by the original DX phenotype. Alternately if cell contact with specific matrices laid down in response to glucocorticoid treatment maintained the low basal levels of ERK activity, initial induction of kinase function would be likely to be maintained for a longer period. Analysis of the time course demonstrating downregulation of ERK activity during monocyte-macrophage differentiation in figure 1, showed ERK activity was decreased after 24hrs but not significantly until day 3-4 which could be interpreted as suggestive of formation of distinct matrix interactions. 5-day DX-treated monocyte/macrophages were detached from tissue culture plastic by

incubation with EDTA on ice, washed, resuspended in IDMEM, and allowed to adhere to virgin plastic for 1, 4 and 6 hrs prior to lysis and assessment of ERK activity. Western blots demonstrated that ERK activation was induced on initial adhesion but decreased within 6 hrs to levels equivalent to that seen in 5-day DX treated cells lysed prior to detachment (fig. 3). This suggests that changes in ERK activity were not due to differential matrix production by DX-treated cells, but dependent on the repertoire of adhesion receptors and/or the downstream signalling proteins employed after DX maturation of monocyte/macrophages.

DX attenuation of basal ERK activity is mediated by decreased expression of C3G and B-Raf

Activation of ERK via soluble mediators, or on engagement of adhesion receptors can be mediated by Ras/c-Raf and Rap1/B-Raf signalling. Crosstalk occurs between the two pathways, as in the absence of B-Raf, Rap1 is antagonistic to Ras function inhibiting ERK activation (Cook and McCormick, 1993). G protein recruitment to receptors/integrins is mediated via adapter proteins, in the case of Rap1 formation of a Crk/C3G complex is sufficient for Rap1 activation during growth factor stimulation and adhesion to matrix. (Buensuceso and O'Toole, 2000; Kao *et al.*, 2001). DX-treatment inhibits expression of C3G (fig. 4). Failure to form Crk/C3G complex could account for the decreased levels of ERK activity after DX-treatment. Analysis of components of Rap1 and other components of this signalling pathway by western blotting of whole cell lysates demonstrated equal expression of Rap1, but a decreased expression of B-Raf after DX-treatment (fig 4). Therefore activation of Rap1 independent of C3G, for example via cAMP or PKA would therefore further inhibit ERK activation (Dugan *et al.*, 1999). This pathway may account for resting levels or ERK activity but is unlikely to be engaged, and more likely inhibited upon "replating".

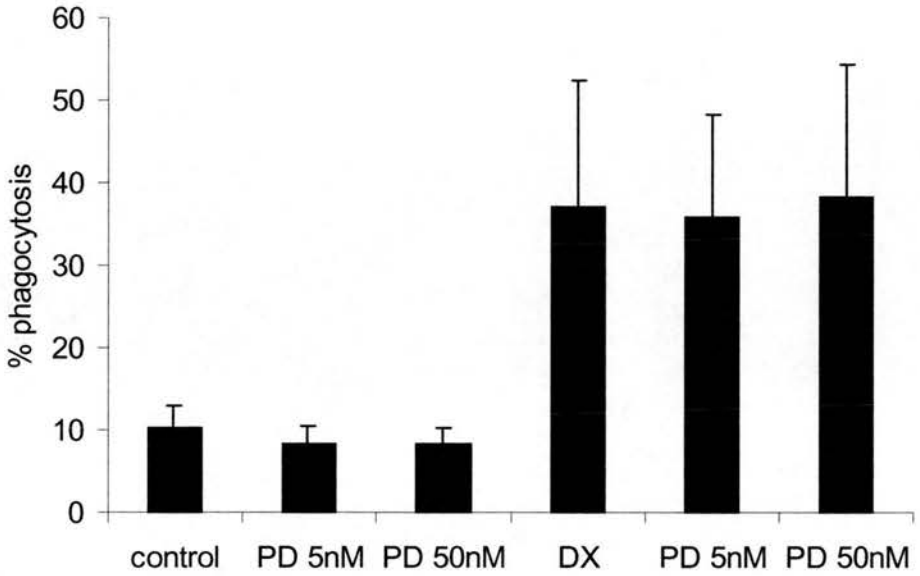


Figure 2. ERK activity is not required for phagocytosis
Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days. Macrophages were pre-incubated with 5-50nM PD98059, for 30 min prior to the assessment of phagocytic capacity for apoptotic neutrophils as described in chapter 1. Data presented as mean phagocytosis, n=3.

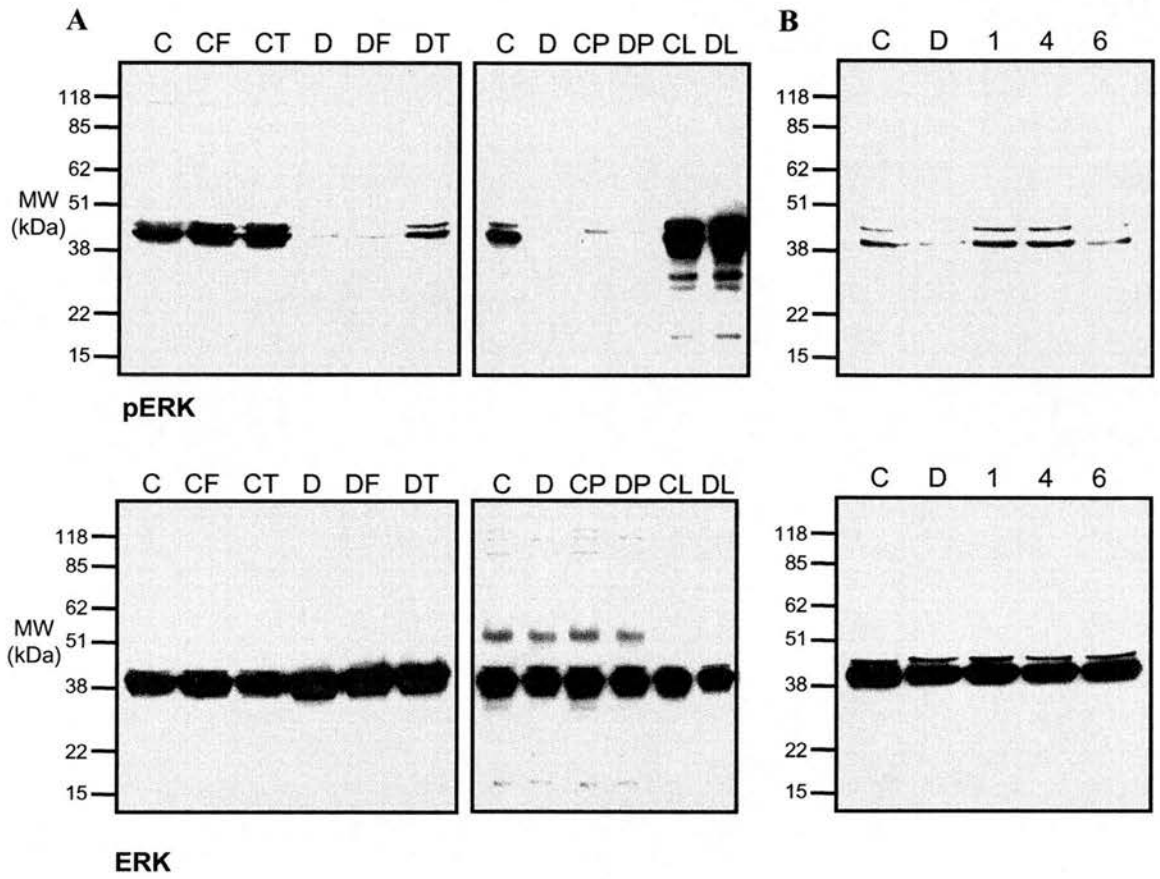


Figure 3. Stimulation of ERK activity in DX treated macrophages

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days. **A** Macrophages were incubated for 30 min with either medium alone, fMLP (1nM), TNF (10ng/ml), PD98059 (50nM), or LPS (1μg/ml), before lysis, and assessment of ERK activity as for figure 1. **B** Macrophages were removed from culture plastic with EDTA, and replated onto virgin plastic. Lysates were made over a time course up to 6 hrs, and ERK activity assessed as for figure 1. C, control untreated cells; D, dexamethasone; F, fMLP; T, TNF; P, PD98059; L, LPS; CF, control plus fMLP etc.

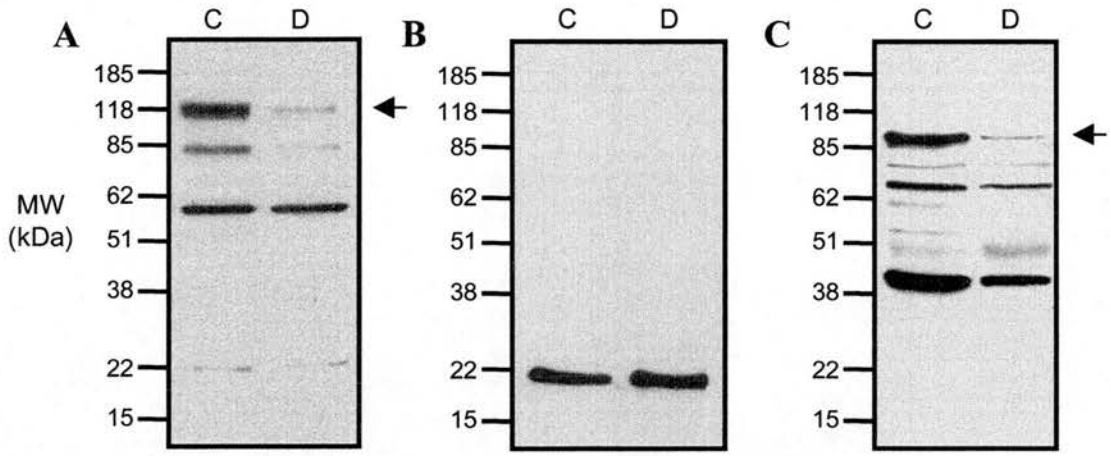


Figure 4. DX alters components of the Rap1 signalling pathway
Peripheral blood monocyte derived macrophages were matured by adherent culture for 5 days ± 1μM DX. Macrophage cell lysates were examined for the expression of **A** C3G (121 kDa) **B** Rap1 (21 kDa), and **C** B-Raf (95 kDa), by SDS-PAGE and western blotting. C, untreated control cells; D, DX-treated. Antibody concentrations: C3G, 1:500; Rap1, 1:1000; B-Raf, 1:100.

DX inhibits macrophage migration in vitro

To determine whether the deficit in basal ERK activity and p130cas expression in DX-treated macrophages affected migration in culture, or if activation of ERK with LPS could rescue the phenotype, we used an *in vitro* "wound healing" assay. Cell movement that occurs in response to an artificial "wound" made by scratching a monolayer of cells with a pipette tip can be assessed by quantification of migration of cells back into the "wounded" areas. Initially we used 48 or 24 well culture plates, however this gave equivocal results, particularly in DX-treated cultures as cells appeared to re-seed in the centre of the wound. We had noted that during culture of monocyte/macrophages in 6 well plates, detached cells, contaminating lymphocytes and cellular debris tended to pool in the centre of the well, thus wounding towards the periphery of the well would present a much cleaner assay. Monocyte/macrophages were cultured in 6 well plates for 5 days ± 1μM DX as described previously. Cells were incubated with either 1μg/ml LPS, 50nM PD98059 or culture medium alone for 30 minutes, the monolayer was then wounded with a pipette tip at intervals over a period of 24hrs, after which cells were fixed and migration into the empty area of the culture assessed microscopically (fig. 5). Untreated monocyte/macrophages migrated into the space with significant movement after 12hrs, and confluency after 24hrs. Incubation of control cultures with LPS which elevates ERK activity did not appear to augment migration, rather migration at each time point appeared slightly reduced compared to macrophages incubated with medium alone. Surprisingly the ERK inhibitor PD98059 had only a very slight effect on migration in both un-treated and DX-treated cultures. PD98059 has been shown to both inhibit and not inhibit fibroblast migration (Cary *et al.*, 1998; Cheresh *et al.*, 1999; Klemke *et al.*, 1997), therefore assessment of the stability of the compound during culture, and direct quantification of the phosphorylation state of ERK 1 and 2 in macrophages used in the assay must be addressed before a requirement for ERK activity for migration in our system is dismissed. Compared to untreated macrophages, migration of DX-treated monocyte/macrophages was reduced. However, cells were able to colonise the wound by 24hrs in medium alone and in PD98059-treated cultures. DX-treated macrophages treated with LPS presented an unusual and unexpected pattern of

migration. There was little cell movement at 6 hrs, however by 12 hrs cells had migrated into the wound such that the density of the cells where the monolayer had been scored was higher than in the surrounding culture. This phenomenon was still apparent at 24 hrs, but cell density in the strip was reduced. This observation suggests that wounding may have created a chemotactic signal, possibly through release of cellular contents of damaged cells. These assays demonstrate that in the presence of an activator of ERK, DX-treated cells were able to undergo directed migration.

In contrast to untreated control populations where cells migrating into the wound had a dendritic like, polarized morphology, migrating DX treated macrophages did not appear polarised, even in LPS treated cultures where migration was more evident. However some cells appeared slightly more irregular in shape compared to the normal DX rounded phenotype, even in the LPS treated cultures. Migration of DX-treated cells suggests that the high levels of active Rac may be sufficient to rescue for the loss of p130cas and allow migration. Additionally the assay suggests that with the appropriate chemotactic stimuli DX-programmed macrophages would migrate as proficiently *in vivo* as their unstimulated counterparts.

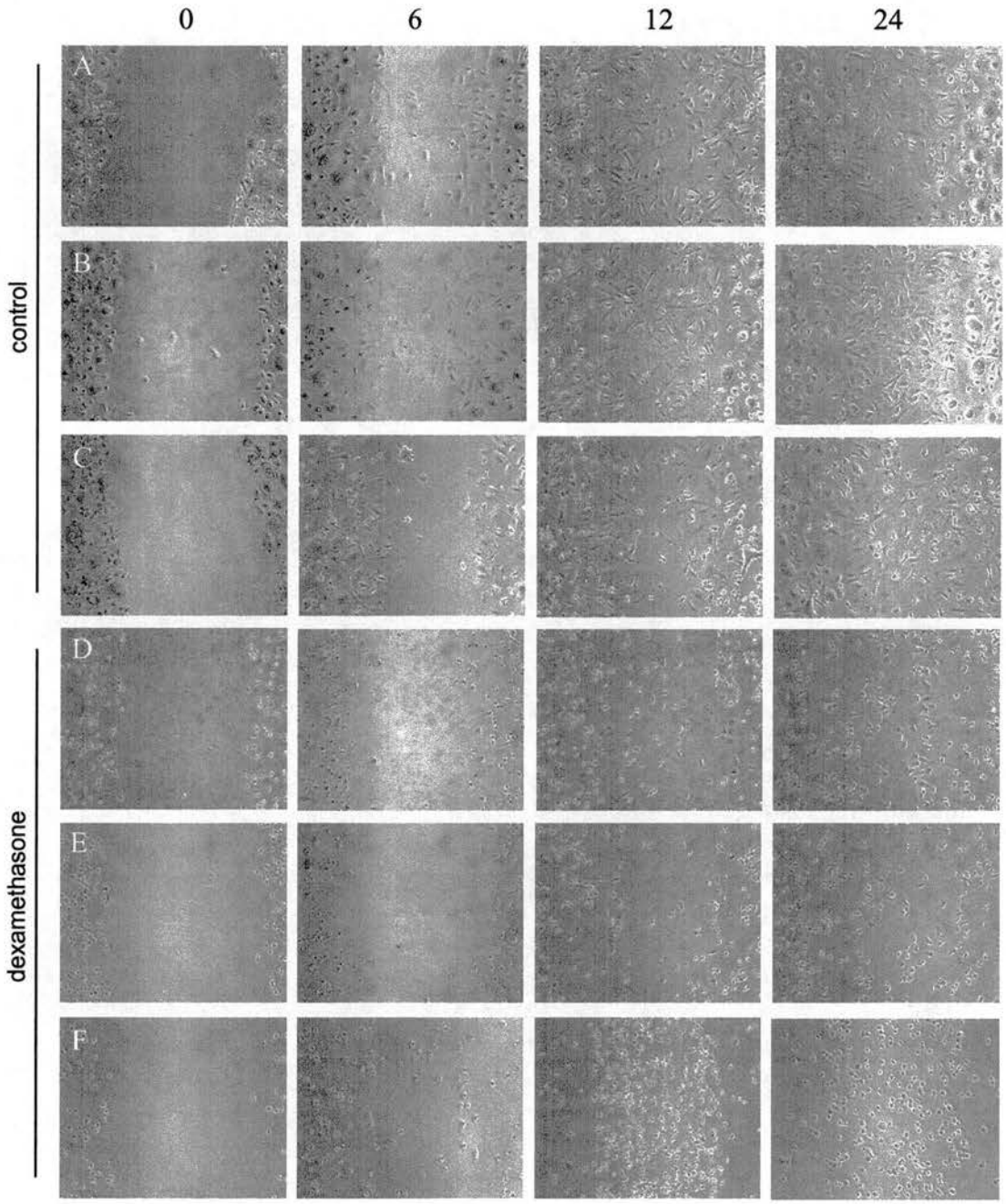


Figure 5. Migration of DX-treated monocyte/macrophages
Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5-days in 6 well culture plates. Control (A, B, C) and DX-treated (D, E, F) macrophages were pre-incubated with either medium alone (A, D), 50nM PD98059 (B, E) or 1μg/ml LPS (C, F). Monolayers were wounded with a pipette tip and fixed at 0, 6, 12, and 24 hrs, and migration assessed by phase microscopy (x10 objective).

Discussion

DX downregulates ERK activity but not motility

DX-treatment of monocytes early during monocyte/macrophage differentiation induces profound morphological and functional changes. DX-treated cells show an increased ability for the rearrangement of cytoskeletal components, a characteristic "rounded" phenotype with a heightened capacity for phagocytosis. One prediction would be that increased cytoskeletal plasticity of DX-treated cells would promote migration. However, DX macrophages are deficient in two components demonstrated to be required for migration in several cell types, the adaptor protein p130cas and activity of the MAPKs, ERK1 and 2 (Cary *et al.*, 1998; Klemke *et al.*, 1997; Klemke *et al.*, 1998). Loss of ERK activity correlated with the downregulation of p130cas expression and the acquisition of morphological and phagocytic phenotypes. It is unlikely that ERK directly regulates phagocytic potential since inhibition of ERK activity in untreated macrophages did not promote phagocytosis. Our inability to detect a role for ERK activity in phagocytosis may be in part due to the nature of the phagocytosis assay used. Macrophage monolayers are covered with a "lawn" of apoptotic cells and may therefore not require active migration, in contrast to the situation *in vivo*.

We have previously demonstrated that down regulation of Pyk2 and paxillin phosphorylation was only obtained if cells were exposed to glucocorticoids early during maturation (see chapter 4). In a similar fashion, we found that ERK activity was dependent on glucocorticoid programming, as ERK activity in cells matured in the absence of DX was refractory to modulation on subsequent steroid challenge. Induction of ERK activity in DX-treated macrophages remained responsive to inflammatory mediators, suggesting that DX did not affect kinase function directly, but altered upstream signalling pathways. Despite a reduction in basal ERK activity, DX-treated macrophages were able to migrate during an *in vitro* "wounding" assay. This may be due to an induction of ERK activity on wounding of the monolayer, compensation of ERK function by other molecules, or induction of movement via an ERK-independent

mechanism. Visualisation of active ERK in migrating cells would determine if ERK were required for DX-treated macrophage migration. Preliminary experiments to visualise active ERK using anti-phospho-ERK mAb produced a diffuse non-specific pattern of staining. The difference in migration rate and pattern of migration between control DX-treated and LPS/DX-treated cultures would suggest that glucocorticoid programmed cells were able to employ mechanisms resulting in random and directed migration. Changes in signalling pathways which may account for the down-regulation of basal ERK activity, or molecules which may compensate for the decrease in kinase function and p130cas expression are discussed below.

Changes in adhesion signalling mediate DX-downregulation of ERK activity

Activation of ERK signalling cascade can occur in response to a number of extracellular stimuli such as growth factors, cytokines, and serum proteins (Schaeffer and Weber, 1999). Many studies have implicated integrins in mediating ERK activation during cell adhesion, as opposed to adhesion receptors such as cadherins, selectins, and the immunoglobulin family of cell adhesion molecules (for review see Aplin *et al.*, 1998). Integrin engagement can potentially induce a number of intracellular signalling pathways (summarised in fig. 6) many of which are able to activate ERK, and influence cellular morphology. The kinetics of ERK activity in DX-treated macrophages after replating onto tissue culture plastic raises a possibility that DX programs a unique repertoire of adhesion receptors that maintain a low basal ERK activity. ERK activity following reattachment may be due to the engagement of a specific receptors which then alters during subsequent culture. Alternatively it has been proposed that ERK can localise to the cytoskeleton (Reszka *et al.*, 1995), and is activated by cytoskeletal dynamics during cell spreading on substratum, or during shape changes under fluid shear stress (Takahashi and Berk, 1996; Tseng *et al.*, 1995). Cytochalasin D, an inhibitor of actin polymerisation, blocked ERK activation in HUVEC cells (Takahashi and Berk, 1996; Zhu and Assoian, 1995). DX treated macrophages do appear to show increased cell spreading upon initial adhesion to "virgin" plastic, but regain a rounded morphology

over time (results not shown), supporting either hypotheses. Determination of the localisation of ERK activity in adhering cells, to either the cytosol or the cytoskeleton, by immunofluorescence staining, or western blotting of cell fractions would allow this to be tested directly. Although we have not investigated the status of all possible activators of ERK activity during DX-treated monocyte/macrophage adhesion and culture in this study, the preliminary data presented here are suggestive that several pathways are compromised by glucocorticoid programming of macrophage differentiation.

Rap1 activation of ERK

G-proteins, Ras and Rap1 can activate similar but distinct pathways leading to ERK activation (fig 6) (for review see Reuther and Der, 2000; Schaeffer and Weber, 1999). Upon integrin engagement, src activation of Pyk2 leads to the recruitment of Ras/Raf via the adaptor protein Grb2, and the Ras-GEF, Sos (Blaukat *et al.*, 1999). Alternatively, Rap1 mediates activation of ERK via B-Raf, and can be linked to adhesion receptors via the Crk/C3G complex (Gotoh *et al.*, 1995; Okada *et al.*, 1998) in conjunction with other adaptor proteins. The specific adaptors have not yet been identified, however Crk is able to bind both paxillin and p130cas in a phosphorylation dependent manner, and C3G has been shown to associate with p130cas *in vitro* (Kirsch *et al.*, 1998; Sakai *et al.*, 1994). Interestingly Crk/C3G were also able to activate ERK activity via a Ras dependent pathway in a haematopoetic cell line 32D, stimulated with soluble mediators erythropoietin or IL-3 (Nosaka *et al.*, 1999). We would predict that C3G down regulation in DX-treated cells would inhibit activation of Rap1 resulting in decreased ERK activity, if this pathway were employed during DX-macrophage adhesion. However, Rap1 activity does not potentiate ERK activity in the absence of B-Raf (York *et al.*, 1998), furthermore association of Rap1 with Ras inhibits Ras activation of Raf-1, decreasing cellular ERK activity (Cook *et al.*, 1993). As B-Raf expression was downregulated in the presence of DX, activation of Rap1 independent of C3G would not be expected to promote ERK activity, and may prevent activation of the kinase by functional Ras pathways. Buensuceso and O'Toole (2000) have demonstrated the importance of B-Raf expression in mediating ERK activation in response to adhesion

signals. Adherent CHO cells had increased ERK activity, low Rap1 activity and no C3G/Crk complex formation. In contrast CHO cells in suspension were shown to have low levels of active ERK, and high levels of active Rap1 from C3G/Crk association. ERK activity was induced in suspension cells by expression of B-Raf. The observation of Crk/C3G coupling in suspension cells conflicts with previous findings that formation of the complex was associated with adhesion in BaF3 cells, a B cell line (Uemura and Griffin, 1999). Signals mediated by the Crk/C3G complex may be cell type specific, or dependent on the Crk family member or C3G splice variant employed (Matsuda *et al.*, 1992; ten Hoeve *et al.*, 1993; Zhai *et al.*, 2001). During cell attachment Crk binds a number of protein partners including p130cas and paxillin, which may sequester Crk from C3G. Loss of p130cas phosphorylation in suspension would disrupt p130cas/Crk complex formation that may promote the interaction of Crk with C3G. The balance of effector proteins present within the cells would therefore determine the complexes formed and hence the functional outcomes. It would be intriguing to know how loss of p130cas and C3G in DX-treated cells effects the pattern of Crk/protein interactions. Regulation of B-Raf expression has also been shown to regulate ERK responses to exogenous stimuli in neighbouring cell types. Central nervous system-derived neurons, but not astrocytes express B-Raf. Consequently elevation of cAMP induces ERK1/2 activity in neurons, promoting cell survival, but inhibits kinase activation in astrocytes, reducing proliferation and promoting differentiation (Dugan *et al.*, 1999). Therefore B-Raf acts as a molecular switch that enables different functional outcomes in cells from a single soluble messenger.

PKA can modulate the activation of Raf-1 and B-Raf. High intracellular cAMP prevents Ras-induced ERK activity by inhibition of Raf-1 function in fibroblasts (Cook and McCormick, 1993; Sevetson *et al.*, 1993). In contrast, elevated cAMP promotes Rap-1 activity (de Rooij *et al.*, 1998; Vossler *et al.*, 1997; York *et al.*, 1998). We have previously shown in the laboratory that treatment of monocyte/macrophages with substances which elevate cellular cAMP, and hence PKA activity, induce rounding and detachment of macrophages resulting in the inhibition of phagocytosis due to the

disruption of cytoskeletal components (Rossi *et al.*, 1998). The morphological appearance and altered actin organisation in DX-treated macrophages is suggestive that intracellular levels of cAMP may be elevated. However, the highly phagocytic nature of DX-treated populations argues against this. The absolute levels of intracellular cAMP and localisation may induce different effects on cell architecture, with a slight elevation inducing cell rounding such as that observed in DX-treated cultures, whereas higher levels may induce disruption of cytoskeletal elements as described by Rossi *et al.* (1998). Increased levels of cAMP, although potentially activating ERK have an opposing effect on the principal ERK substrate during cell migration, MLC-kinase (MLCK). PKA phosphorylates and inhibits MLCK enzymatic activity (Lamb *et al.*, 1988), and hence MLC phosphorylation. In addition, PKA reduces RhoA activation of Rho-kinase, which is also able to promote MLC phosphorylation (Dong *et al.*, 1998; Lang *et al.*, 1996). We would therefore predict that elevated levels of PKA activity would be inhibitory to migration.

ERK activation via Src-kinases and Pyk2

Although FAK is a major mediator of integrin-induced ERK activity, we do not detect expression in monocyte/macrophages. Instead, several FAK functions are mediated by the sister kinase Pyk2. Integrin engagement and src activation induces the recruitment and phosphorylation of Pyk2 to sites of focal contact and membrane ruffles at the leading edge of migratory cells (Duong and Rodan, 2000; Watson *et al.*, 2001; Williams and Ridley, 2000). Changes in Pyk2 phosphorylation may be mediated by an increase in phosphatase activity or decrease in kinase function. Peritoneal exudate macrophages from $Hck^{-/-}/Fgr^{-/-}$ double mutants exhibit decreased tyrosine phosphorylation of paxillin and Pyk2 (Suen *et al.*, 1999). Therefore Src-family kinases represent potential targets for steroid modulation. Phosphorylation of tyrosine residues on Pyk2 enables Ras-mediated ERK activity via Grb2 and Sos (Blaukat *et al.*, 1999). Src can also initiate ERK activation independent of Pyk2 with direct localisation of Grb2/Sos via the adaptor protein Shc, which has been linked to the regulation of random cell migration (Collins *et al.*, 1999; Gu *et al.*, 1999). Loss of Pyk2 phosphorylation in resting DX-treated cells

could also potentially compromise ERK activity. Williams and Ridley (2000) demonstrated that exposure of peripheral blood monocytes and macrophage cell lines to LPS induced phosphorylation of paxillin and Pyk2. We have not assessed the effect of LPS treatment on paxillin or Pyk2 phosphorylation in DX-treated cells, but we do observe augmented ERK phosphorylation. Since replating DX-treated macrophages induced both ERK and paxillin phosphorylation we would predict increased Pyk2 phosphorylation on reattachment. I therefore propose that Pyk2 dephosphorylation observed during DX-programming may be the principal effector for the decreased ERK phosphorylation observed in glucocorticoid-treated cells. The use of pharmacological inhibitors of Pyk2 phosphorylation would provide a useful tool for the investigation of this pathway.

ERK activation via Rho family GTPases

Cross talk between signalling pathways has revealed induction of ERK activity by Rho family GTPases. Rho has been proposed to activate ERK activity via interaction with Raf-1 (Li *et al.*, 2001), additionally Cdc42 and Rac activate the ERK cascade, via PAK1. PAK1 phosphorylates MEK on ser298 required for the maximal association of MEK with Raf-1 (Frost *et al.*, 1997). However PAK1 can also phosphorylate and inactivate MLCK (Sanders *et al.*, 1999; van Leeuwen *et al.*, 1999). Localisation of opposing signals may allow the inhibition of contraction during cell protrusion at the leading edge, but allow contraction in the tail. Despite high levels of active Rac in DX-treated cells we see no resting ERK activity. Potentiation of MAPK signalling pathways by Rho-family GTPases in DX-treated cells may require further molecular cues not present in these cells, alternatively Rac may be sequestered away from components of the MAPK pathway. Interestingly, overexpression of active Rac in either tumour cell lines or p130cas deficient fibroblasts was not sufficient to promote migration (Cho and Klemke, 2000). This suggests that additional functions mediated by the p130cas/Crk complex are required for cell movement, for example specific localisation of active Rac to the leading edge of the cell. Localisation of Rac and phosphorylated ERK by

immunofluorescent staining may suggest if Rac is able to influence ERK activity in migrating DX-treated macrophages.

PI 3-Kinase and ERK activation

PI 3-kinase activity has been shown to be important for cell polarization during directed migration (Sasaki *et al.*, 2000; Servant *et al.*, 2000). The lipid kinase has also been shown to activate ERK activity in response to adhesion, via a multi-protein complex containing the tyrosine kinase syk, adaptor proteins cbl and Grb2, and the GEF Vav1, in response to $\alpha_{IIb}\beta_3$ integrin ligation (Miranti *et al.*, 1998). Additionally, PI 3-kinase coupling to the adaptor protein cbl, has been shown to maintain basal ERK activity in HL60 cells (Finkelstein and Shimizu, 2000). Preliminary evidence suggested that tyrosine phosphorylation of cbl and Vav, required for protein/protein interactions is downregulated in DX-treated macrophages (results not shown), which could compromise directed migration. PI 3-kinase can promote movement independent of ERK activation through Rho-GTPases (Rickert *et al.*, 2000). The catalytic domain of PI 3-kinase facilitates Rac activation, lamellipodia formation, membrane ruffling, and migration towards a CSF-1 gradient (Vanhaesebroeck *et al.*, 1999). Additionally the p85 subunit is required for Cdc42 induction of filopodial formation during fibroblast migration towards PDGF (Meng and Lowell, 1998). In addition to its role in regulating cytoskeletal organisation, PI3-kinase has been implicated in the regulation of exocytic insertion of recycling endosomes at the leading edge of the cell, required for the extension of cellular processes during phagocytosis (Bajno *et al.*, 2000). A similar requirement for recycling of membrane has been proposed for migration (Mellman, 2000). Recycling receptors have been shown to appear preferentially at forward margins of migrating cells (Pierini *et al.*, 2000).

Directed verses random migration: Migration of DX-treated macrophages

The "wounding" assay provides a simple assessment of cell motility, and provides an assay for directed movement. Scoring of the plate may induce the active release of soluble mediators from damaged cells that could function as a chemoattractant. Movement after scoring a monolayer of cells may be induced by loss of cell-cell contact, with movement into the gap, until contact with neighbouring cells occurs again, as demonstrated by macrophages in untreated cultures. DX treated cells were able to colonise the gap left by scoring, although movement appeared to be less efficient than untreated cells, with only a slight polarization of cells during migration, suggestive of random movement. Interpretation of the "type" of motility induced in either culture must be made with caution. The cell density of the DX-treated cells was lower than untreated cells, with very few contacts between neighbouring cells in the culture. Although apparent to a greater degree in this assay, it is a feature of all DX-treated macrophage cultures. The lack of cell-cell contact might prevent DX-treated macrophages "sensing" the wounded area. Direct comparison with untreated cultures may only be valid with a complete monolayer of DX treated cells. In view of this, re-colonisation of the wound to a level nearly comparable to original cell density after 24hrs by random movement suggests DX-treated macrophages may in fact be more motile than their untreated counterparts. Time laspe video microscopy would reveal the role of directed or random migration in the re-colonisation process.

Migration of cells in the presence of LPS produced some intriguing results. Control cells appeared to migrate slightly less than non-LPS-treated cultures, despite the induction of elevated levels of ERK by LPS. Williams and Ridley (2000) demonstrated that LPS treatment of the macrophage cell line J774 induced phosphorylation of paxillin and Pyk2 and increased cell spreading. Augmentation of adhesive contacts could decrease migration. Migration of DX-treated cells in the presence of LPS was suggestive of a directional response, as the density of cells in the wound was greater than in the surrounding culture after 12 hours. Cell density in the wound decreased after 24hrs suggesting a resumption of random migration by glucocorticoid programmed

cells. In the absence of p130cas, we would not predict an induction of directional migration (Cary *et al.*, 1998; Cheresh *et al.*, 1999; Klemke *et al.*, 1998; Rigot *et al.*, 1998; Yenush *et al.*, 1994). However, some groups have demonstrated that p130cas is not required for directed migration (Bornfeldt *et al.*, 1995; Coffer *et al.*, 1998). The high levels of active Rac observed in DX-treated macrophages, which is thought to be functionally downstream of p130cas in migration signalling (Cheresh *et al.*, 1999), may be sufficient for cell movement in the absence of p130cas expression, and may allow rapid movement when ERK activity is elevated by LPS. The lack of inhibition of movement in either untreated or DX-treated cells by PD98059 would suggest ERK activity is not required for directed or random migration in our system. Analysis of DX-treated cell migration in the presence of LPS (and hence a strong ERK response) and PD98059 may be more informative. Assessment of the activity of PD98059 during the time course of the assay must be verified before conclusions are drawn. It would be naïve to expect migration would be regulated by one single pathway across a broad range of cell types and exogenous stimuli. Cell type and stimuli specific pathways would ensure that, for example, an inflammatory cytokine, which may be perceived by a number of inflammatory cells would only be translated into movement by a subset. Modulation of a number of signalling pathways by DX has been discussed above. It is unlikely that all the pathways modulated by DX-programming will have a direct functional effect on DX-treated macrophage movement, but may affect other cellular processes that also utilise components of that signalling pathway.

Wider implications

Engagement of specific adhesion receptors, or cytokine/growth factor receptor stimulation, can induce a number of intracellular signalling pathways leading to ERK activation. The specific pathway employed is able to determine the duration and amplitude of ERK activity, and subsequently the biological outcome. For example Epidermal growth factor receptor (EGF-R) stimulation of neuronal PC12 cells induces transient activation of ERK 1 and 2 activity inducing a proliferative response whereas treatment of cells with NGF promotes a sustained activation of ERK 1 and 2 leading to

neuronal differentiation. The sustained as opposed to transient activation has been suggested to be due to activation via Rap1-B-raf interaction on NGF R stimulation as opposed to EGF R recruitment of Ras-c-Raf (York *et al.*, 1998). The duration and amplitude of the ERK response may determine the functional outcome by activation of transcription factors such as Elk-1 and Ets. Ets induces the transcription of a number of proteins required for proliferation, p21, cyclinD1, and Fos. As macrophages are terminally differentiated cells, decrease in mitogenic signals will have no effect on proliferation but may affect cell viability. Cho and Klemke (2000) have proposed that migratory cells suppress their apoptotic pathway during migration and detachment from matrix via p130cas/crk and ERK pathways. DX downregulation of both p130cas and basal ERK activity may have implications for monocyte/macrophage survival during migration *in vivo*. The induction of ERK activity with LPS and TNFα would suggest that DX-programmed cells are able to respond to some soluble mediators. However, ERK activity has been shown to be required for the active secretion of TNFα (Barouch *et al.*, 2001; Kraatz *et al.*, 1999; Means *et al.*, 2000), and some cellular effects of IFNγ are thought to be mediated via the C3G/Rap1 signalling pathway in human NB-4 cells. How DX-programmed macrophages respond and interact with their cytokine environment is an important question and will be addressed in results chapter 7.

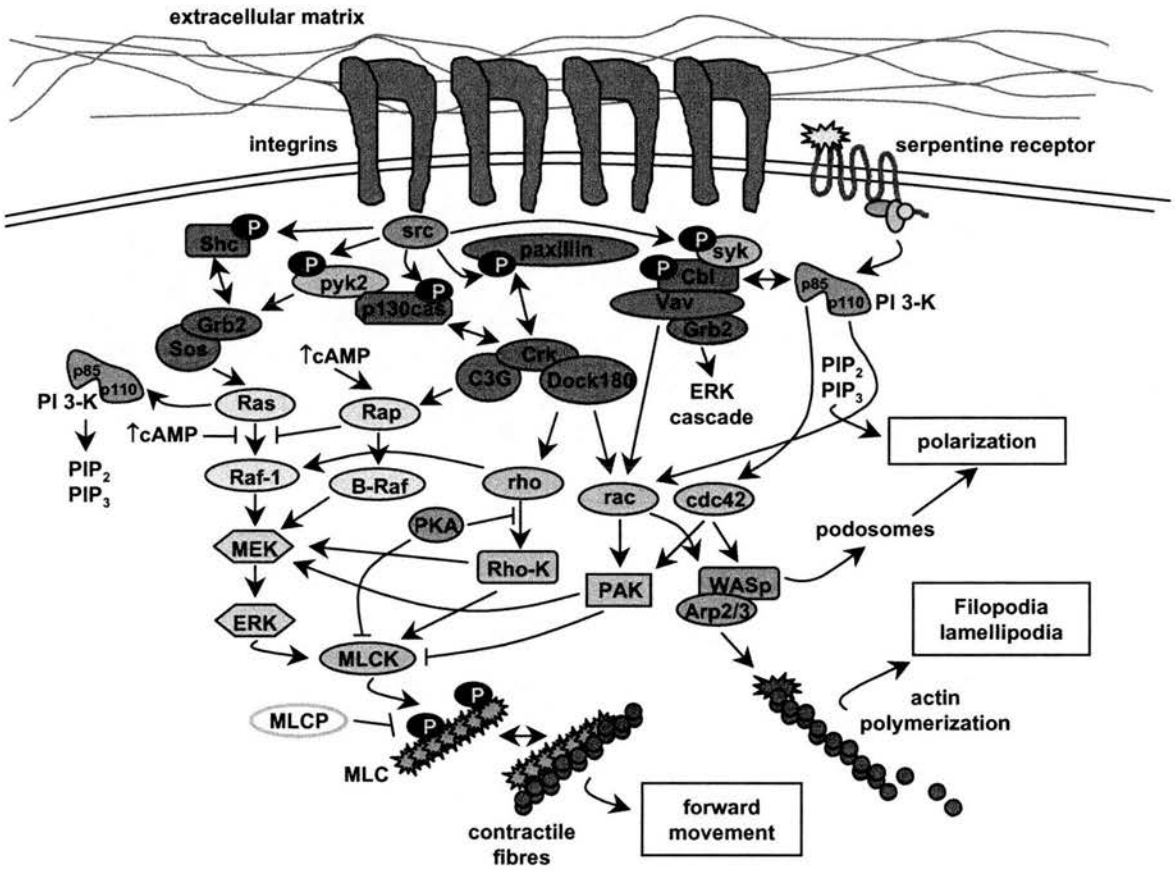


Figure 6. ERK and Rho-GTPase signalling pathways during migration
Adhesion to matrix or occupancy of chemokine receptors activates a number or pathways leading to migration. PI 3-kinase and its lipid products induce cellular polarization, Cdc42/Rac and WASp/Arp2/3 actin polymerisation mediates extension of membrane processes, and activation of the ERK signalling cascade results in contraction of the cell through phosphorylation of MLC and formation of actomyosin fibres. See text for details.

CHAPTER 6: GENERATION OF AN HIV TAT FUSION PROTEIN FOR THE TRASNDUCTION OF P130CAS INTO MACROPAHGES

Introduction

Based upon data presented in chapters 4 and 5, we have proposed that glucocorticoids "reprogram" macrophage differentiation resulting in a phenotype characterised by altered cytoskeletal organisation, and augmented phagocytic capacity. Our results suggest that this is mediated by the changes in expression and activation of a number of proteins previously shown to regulate cytoskeletal dynamics and which are also components of the phagocytic machinery. In particular, DX down regulates the expression of p130cas, an adapter molecule involved in a number of cellular functions including migration, adhesion, cell cycle, apoptosis, and recently phagocytosis (Albert et al., 2000; O'Neill et al., 2000). We hypothesised that down regulation of p130cas expression promotes the reduction of adhesion structures resulting in an increased availability of cytoskeletal components required for phagocytosis. Re-expression of p130cas in DX-treated macrophages would allow us to directly assess the importance of this protein in DX-programming. However, transfection of macrophages with DNA expression constructs is exceedingly inefficient. Lipid based transfection protocols and electroporation result in only a low percentage of transfected cells, although some success has been achieved using adenoviral vectors (Foxwell et al., 1998). One potential problem is that adenoviral entry is mediated in part via $\alpha_v\beta_3$ integrin, a putative "phagocytic receptor". In addition, p130cas phosphorylation has been shown to be required for adenoviral entry into colon carcinoma cells (Li et al., 2000). The use of adenoviral vectors to study the effects of p130cas expression on phagocytic processes may therefore produce equivocal results. Microinjection of plasmid constructs or purified protein has also been used for expression of proteins in macrophages. Since this procedure requires the direct physical introduction of material into cells, only a few percent of cells in a culture can be modified, prohibiting this method for biochemical analysis or studies which require responses from the cell population as a whole. An

alternative approach would be the use of cell lines. Treatment of the mouse macrophage line J774 with DX induced a similar down regulation of p130cas expression as is seen in human peripheral blood monocyte derived macrophages. However, phagocytosis of apoptotic cells in both untreated and DX-treated J774s was very low (~ 1%), and therefore this does not present as a viable model.

Direct introduction of proteins into a variety of cell types including macrophages has recently been achieved by utilising peptide domains that enable the direct transduction of proteins through the cell membrane. Transduction mediated by the HIV TAT protein was first demonstrated independently by Green and Frankel when TAT was added to culture media (Frankel and Pabo, 1988; Green and Loewenstein, 1998). The ability of TAT to transduce into cells is dependent on an eleven amino acid sequence termed the protein-transduction domain (PTD). PTDs have also been identified in Antennapedia (Antp) (Joliot et al., 1991), a homeotic transcription factor of *Drosophila*, and the herpes-simplex-virus-1 DNA binding protein VP22 (Elliott and O'Hare, 1997). The mechanism of transduction is unknown, however it does not require a classical receptor, transporter, endosome, or absorptive-endocytosis-mediated process (Derossi et al., 1996; Derossi et al., 1994; Elliott and O'Hare, 1997; Mann and Frankel, 1991; Vives et al., 1997), and efficient transduction of Antp and TAT still occurs at 4°C (Derossi et al., 1994; Vives et al., 1997). Sequence analysis revealed the presence of basic residues within the PTD which may facilitate contact with negatively charged lipids (Elliott and O'Hare, 1997; Green and Loewenstein, 1998; Joliot et al., 1991). Secondary structure analysis also offers few clues to the transduction mechanism. Antp PTD when translated as part of the whole protein demonstrates a helical formation, however the Antp PTD peptide in isolation is less structured (Derossi et al., 1996). Additionally TAT PTD appears structure-less by circular dichroism and nuclear magnetic resonance spectroscopy (Loret et al., 1991; Mabrouk et al., 1991), but protein-structure predicting algorithms suggest that TAT PTD can adopt a helical conformation (Schwarze et al., 2000). It is not known if VP22, TAT or Antp transduce via a common mechanism. Antp induced transduction is limited to proteins with less than 100 residues (Derossi et al., 1998), whereas both TAT and VP22 PTDs are able to allow entry of molecules of over 1000 amino acids

(Fawell et al., 1994; Schwarze et al., 1999). Moreover superparamagnetic iron particles up to 40nm in diameter, coated in TAT PTD have been introduced into T cells (Lewin et al., 2000). A model of Antp transduction involving inverted micelles has been proposed by Derossi et al. (1998), in contrast Dowdy and colleagues suggest in the instance of TAT, a mechanism whereby the localised positive charge of the PTD directly penetrates the lipid bilayer (Schwarze et al., 2000). Whatever the mechanism of transduction, proteins appear to be partially or totally unfolded during entry, with refolding inside the cell by HSP90 heat shock proteins (Bonifaci et al., 1995). This can result in loss of activity in certain cases, for example transduction of TAT-GFP into mammalian cells results in a high intracellular level of TAT-GFP, but significant loss of GFP fluorescence (Schwarze et al., 2000). The efficiency of correct refolding is likely to be protein specific, and may be problematic when transducing proteins across species. In order to test our hypothesis that p130cas downregulation is an important factor in mediating DX reprogramming we constructed a TAT-p130cas fusion protein, plus a TAT-βgalactosidase fusion as a control for transduction into DX-treated monocyte-macrophages. The fusion protein was created by insertion of sequence into the pTAT-HA vector (kindly supplied by S.F. Dowdy, Washington University School of Medicine, St. Louis, USA). This construct contains an N-terminal 6-histidine leader, for affinity purification, followed by the 11 amino acid TAT PTD, flanked by glycine residues (for free bond rotation of the domain), and a hemagglutinin (HA) tag, under the control of an IPTG inducible T7 promoter.

Cloning Strategy

Full-length human p130cas was kindly provided by F. Hildebrant (University Children's Hospital, Freiburg, Germany), in pUNI-V5-His vector (Invitrogen). The sequence was identical to accession nm_014567 with the exception of two silent polymorphisms at position 1384: g=>a, and 2065: a=>g. The pUNI-V5-His/p130cas construct was originally generated by T/A cloning of PCR amplified p130cas (coding region nucleotides 122-2734 of nm_014567, no 3' UTR), consequently no restriction enzyme sites for excision of the coding region were available. Construction of HIV-

TAT fusion protein requires an in-frame insertion of coding sequence into the NcoI restriction enzyme site located 5' to the TAT peptide sequence, minimising the insertion of "junk" amino acids which may compromise the folding or function of the resulting fusion protein. Our initial strategy was to design a 5' primer corresponding to sequence encoding the first few amino acids of p130cas, but with the insertion of an artificial NcoI site around the ATG initiation codon. Secondly, we would design a 3' primer for annealing to pUNI-V5-His vector sequence directly after the T/A cloning site, generating an artificial XhoI restriction site for ligation into the final TAT vector, removing any 3' UTR from the pUNI-V5-His vector. The resulting PCR product would be sub-cloned into pGEMT vector (Promega) via A overhangs, excised via NcoI/XhoI digest, and ligated into the pTAT construct. However p130cas contains two NcoI sites (positions 711 and 933 of nm_014567 sequence), digestion of pGEMT/p130cas or the p130cas PCR product directly would result in fragmentation of the coding sequence, and failure to clone the full length insert. We therefore employed an alternative strategy whereby p130cas was amplified by PCR using the original 3' primer, but with a second 5' oligomer containing an artificial KpnI restriction site upstream of the p130cas coding sequence. This would allow in-frame ligation of p130cas into pTAT-HA vector six base-pairs 5' to the original NcoI cloning site, with insertion of four additional amino acids (alanine, glycine threonine and methionine) between the TAT and p130cas sequences.

Results

PCR amplification of p130cas

Full-length p130cas was amplified from pUNI-V5-His/p130cas vector using the proofreading *Pwo* DNA polymerase and the following primers:

Forward: $^{5'}$AAA CCG **GGT** ACC *ATG* AAC CAC CTG AAC GT $^{3'}$

Reverse: $^{5'}$ GGC TTA CCG AGC TC**G AG**A ATT GCC CT $^{3'}$

The inserted KpnI restriction site GGTACC (underlined) resulted in the substitution of two base pairs, AC=>GT (bold), 5' to the ATG start (italics). The reverse primer was designed from vector sequence immediately down stream of the PCR cloning

site of pUNI-V5-His. This produced a substitution of two base pairs CC=>GA (bold) creating a XhoI restriction site CTCGAG. A product of ~2.7 kb was amplified using an annealing temperature of 60°C, corresponding to the 2.62 kb coding region of p130cas (Fig 1a).

Cloning p130cas PCR product into pGEM-T easy vector

Pwo was used for amplification of p130cas due to its proofreading capacity, however this enzyme does not produce poly-A overhangs at the ends of PCR products, in contrast to *Taq* polymerase. PCR product was therefore incubated with dATP and *Taq* polymerase, to "tail" the product for ligation into pGEM-T. Concentration of product was estimated visually by running an aliquot on a 1% agarose gel, and the remaining product ligated into pGEM-T easy vector (Promega) at a 3:1 (insert:vector) ratio. Ligations were transformed into *E.coli* (TOP10 strain), plated onto agarose containing ampicillin and X-gal/IPTG for blue/white selection of positive clones. Insertion of PCR product into pGEM-T easy vector disrupts coding of the β-galactosidase (βgal) encoding *LacZ* gene, positive clones are unable to metabolise the chromogenic substrate X-gal, and colonies remain white. Half a dozen positive colonies were picked and amplified by growth overnight in liquid culture plus ampicillin selection. Plasmids were extracted using a Promega Wizard mini-prep kit, and the resulting plasmid DNA checked for inclusion of the p130cas sequence by restriction digest with EcoRI. EcoRI restriction sites flag the PCR cloning site, "dropping-out" the insert resulting, in two bands of approximately 3.0 kb (pGEM-T), and 2.7 kb (p130cas) (fig. 1b).

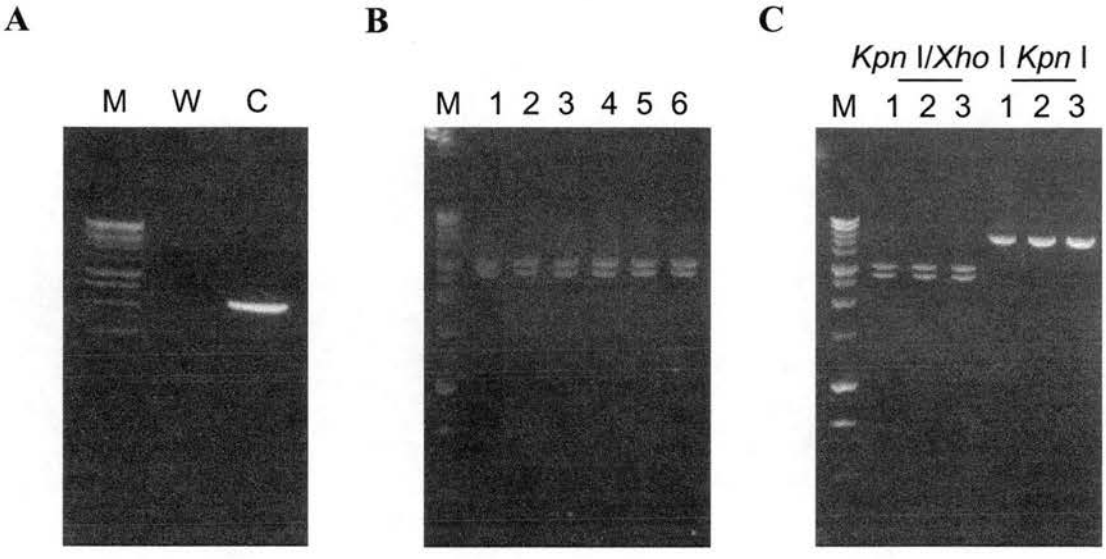


Figure 1. Construction of pTAT/HA-p130cas

A PCR amplification of the 2.7 kb coding region of p130cas from pUNI-V5-His/p130cas (gift from F. Hildebrant, University Children's Hospital, Freiburg, Germany). Forward primer AAA CCG GGT ACC ATG AAC CAC CTG AAC GT, Reverse primer GGC TTA CCG AGC TCG AGA ATT GCC CT, annealing 60°C. **B** Mini-prep DNA of pGEM-T-p130cas sub-cloning digested with EcoRI. Bands correspond to insert (2.7 kb), and plasmid (3.0 kb). **C** Mini-prep DNA of pTAT/HA-p130cas sub-cloning digested with KpnI and KpnI/XhoI. Bands correspond to linearised vector (5.7 kb), insert (2.7 kb), and plasmid (3 kb). M, marker, corresponding to 1 kb ladder (10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25 kb); W, water control; C, p130cas; 1-6, individual transformants.

Sub-cloning p130cas into pTAT/pTAT-HA

After creation of pGEM-T-p130cas, we next subcloned the p130cas sequence into pTAT/HA using the artificial restriction sites engineered during PCR amplification. pGEM-T-p130cas and pTAT/HA were digested with KpnI, an aliquot of the digest visualised on an agarose gel to ensure full linearisation had occurred, and the remaining DNA purified before digestion with XhoI, as the two enzymes required different salt buffers for optimal activity. After digestion with XhoI, pGEM-T and p130cas fragments were separated on an agarose gel, and the 2.7 kb band corresponding to p130cas was excised and purified. As digestion of pTAT vectors produces only one major product, plasmid was purified directly from the digest. The pTAT-HA-p130cas was created by ligation of products in a 3:1 insert:vector ratio and transformed into TOP10s. The pTAT vector does not support blue/white selection therefore in order to estimate the efficiency of the ligation reaction, a second ligation containing vector only was set up in parallel to pTAT-HA-p130cas, and equal amounts of bacterial transformants plated onto agar overnight. Comparison of the number of colonies between vector only and vector plus insert, then allowed estimation of the percentage of positive clones. pTAT-HA-p130cas produced approximately a 5 fold greater number of colonies than the vector only plates. Ten clones of the pTAT-HA-p130cas transformants were picked, amplified overnight in liquid culture, plasmid extracted and assessed for presence of insert by digestion with BamHI and SphI. This combination of restriction enzymes "dropped out" the p130cas insert in 3/12 clones (results not shown). Sequential digestion of the three positive clones demonstrated insert of the plasmid at KpnI and XhoI sites as expected, producing bands of 5.7 kb (linearised vector), 2.7 kb (p130cas insert) and 3.0 kb (pTAT vector) (fig. 1c).

Sequencing pTAT-HA-p130cas construct

Although a proofreading polymerase enzyme was used for amplification of p130cas from the gift vector, sequence changes may still have occurred. We therefore designed 8 primers to allow sequencing of the entire 2.65 kb reading frame of p130cas in the pTAT-HA-p130cas construct. Plasmid DNA from clone 1 was

retransformed into TOP10s and plated onto agar with ampicillin selection. A single colony was picked and grown in 50mls of liquid culture for bulk purification of DNA, using a Qiagen plasmid midi prep kit, resulting in purification of 100µl of plasmid DNA at 2.1µg/µl. Plasmid was then sequenced using an automated ABI sequencer by N. Kotelevtseva (CVRI Molecular Physiology Group, Edinburgh University). The sequence of the TAT-HA-p130cas coding region, plus positions of sequencing primers is shown in Appendix A. Cloning resulted in an in-frame fusion, however four base pair substitutions had occurred during PCR amplification of the p130cas transcript, at positions 390 (A→G), 476 (A→G), 1501 (A→T), and 2364 (T→A) (corresponding to sequence accession number nm_014567), resulting in His→Arg; Thr→Ala; Gln→Leu; and Leu→Gln amino acid substitutions respectively (see Appendix A).

Construction of pTAT-HA/LacZ control plasmid

We would predict that transduction of TAT/p130cas fusion protein would affect cell morphology, and possibly phagocytic function. In order to determine if changes in macrophage function are specific for the re-expression of p130cas and not the introduction of "foreign protein" per se, we constructed a control vector containing the *LacZ* gene which encodes the β-galactosidase (βgal) enzyme. βgal at 119 kDa is of a comparable size to p130cas, is non toxic, and has the ability to produce blue pigmentation when incubated with the chromogenic substrate X-gal, enabling estimation of transduction efficiency by light microscopy.

A vector containing sequence encoding β-Gal, pnlacF, kindly supplied by A. Lacy-Hulbert (CIR, Edinburgh University), was first digested with BglII , the resulting 5' overhangs blunted using Klenow DNA polymerase, and further digested with NcoI, producing a fragment of approximately 3.6 kb. This corresponds to the 3.06 kb coding region plus downstream sequence from the pnlacF vector. βgal was then ligated into pTAT-HA via 5' NcoI, and 3'XhoI (blunted) restriction sites. Ligations were transformed into TOP10s as before, plating on ampicillin plates containing X-Gal/IPTG for blue/white selection. Blue colonies were picked and screened by

NcoI/SphI digest which "dropped out" the insert in 6/6 clones (fig. 2). The blue white selection also demonstrated the transcript was cloned "in-frame", producing functional β-galactosidase.

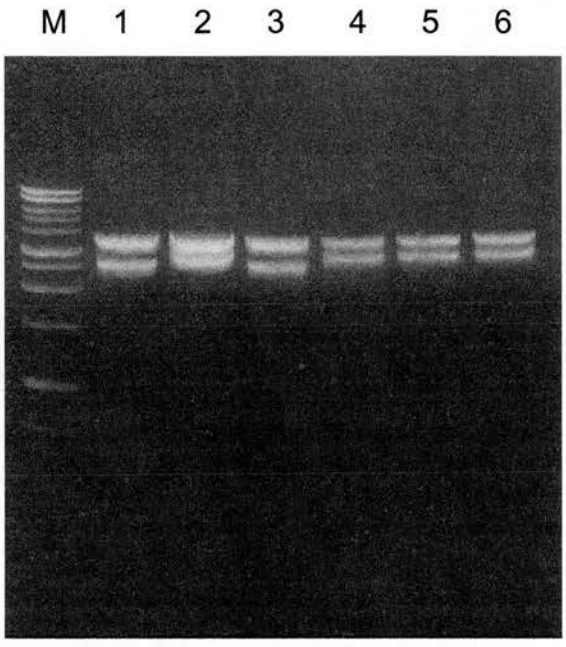
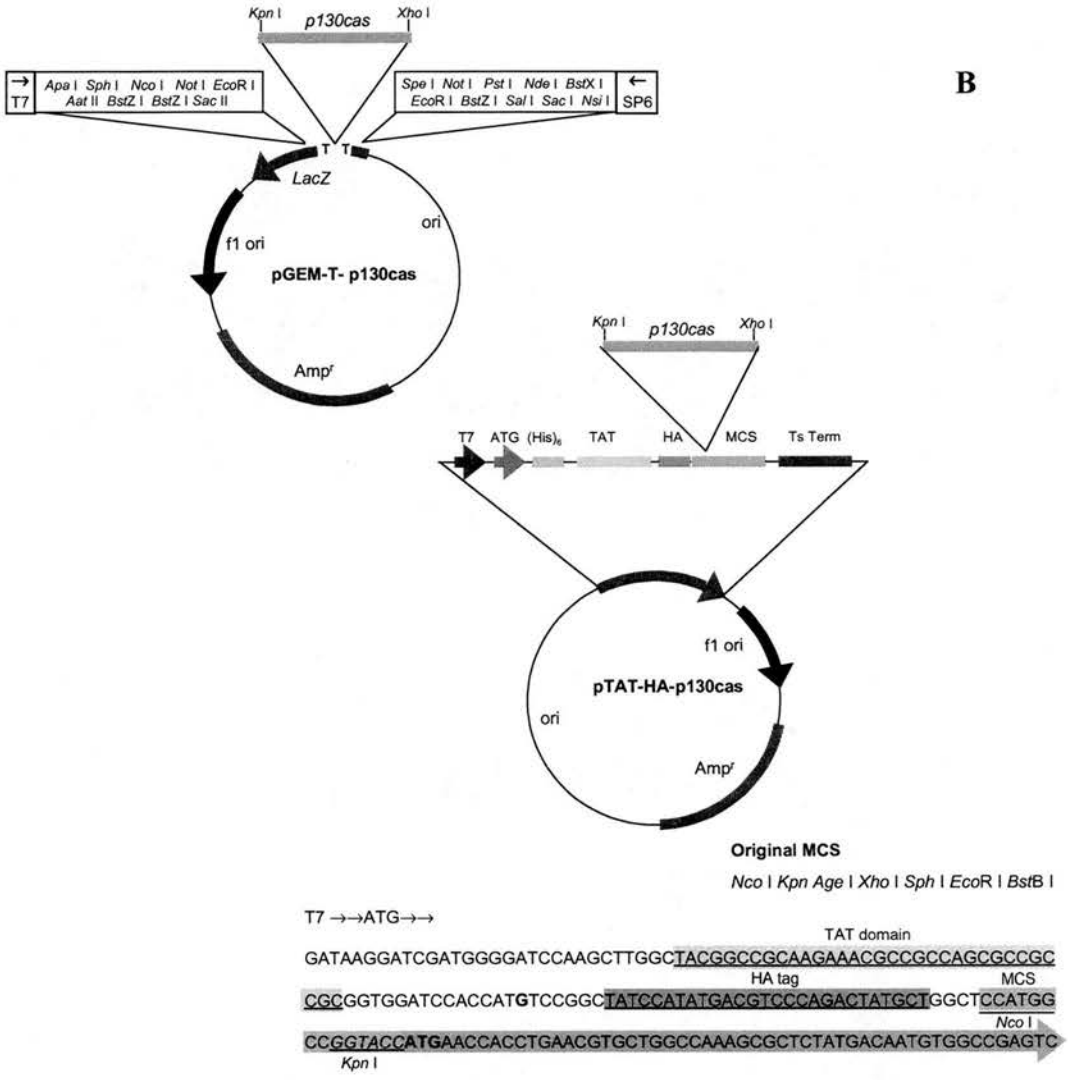


Figure 2 . Construction of pTAT/HA-βgal
Mini-prep DNA of pTAT/HA-βgal sub cloning, digested with NcoI/SphI. Bands correspond to insert (3.6 kb) and vector (3.0 kb). M, marker, 1kb ladder; 1-6, individual clones.



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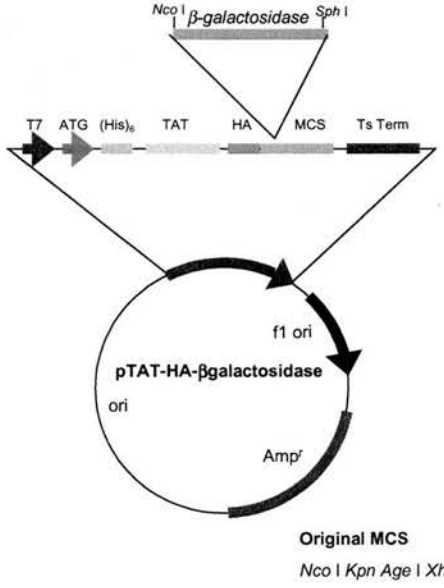

Figure 3. Plasmid constructs
Schematic representation of
A pGEM-T-easy-p130cas
B pTAT-HA-p130cas
C pTAT-HA-βGal

Production of TAT fusion protein: Selection of high expressing clones

Fusion proteins can be produced in *E.coli* by transfection of a plasmid construct containing the gene of choice. The pTAT plasmid contains an IPTG inducible promoter. However upon induction, not all transformants will synthesise protein at the same level. This can be due to the copy number maintained by the clone, but can also be affected by the toxicity of the protein. Additionally, production is likely to have an inverse correlation with growth and replication, with very high levels of protein production compromising viability. We therefore assessed the level of p130cas and βgal synthesis from a number of individual colonies.

pTAT-HA-p130cas and pTAT-HA-βgal were transformed into *E.coli* strain BL21, plated onto ampicillin plates and 6 colonies from each transformation selected and grown as starter cultures O/N in the presence of antibiotic selection. The following day starter cultures were used to inoculate media for growth of cells to an OD_{600} of 0.5, before induction of protein synthesis for 5hrs with IPTG. Cells were lysed and protein solubilised using lysis buffer containing 8M urea. Initially samples were analysed immediately by SDS-PAGE then either Coomassie-blue protein staining of acrylamide gels, or by western blotting with mAb specific for the HA epitope or p130cas. Bands of approximate size could be identified by Coomassie-blue staining, with 2/6 pTAT-p130cas transformants, and 3/6 pTAT-βgal transformants appearing to express fusion protein to a detectable level. To ensure the candidate bands corresponded to p130cas and βgal fusions, samples were assessed by western blotting. Unfortunately both HA and p130cas mAb had a high level of cross reactivity with endogenous bacterial proteins, masking identification of fusion proteins. To minimise the presence of cross reacting protein, the clones viewed as high expressers by Coomassie-blue analysis, were grown and induced as before, but fusion proteins precipitated with a small amount of Ni-NTA resin, prior to analysis by SDS-PAGE and western blotting. Semi-purification and concentration of the fusion proteins allowed clearer identification by Coomassie-blue staining and enabled definite identification when a sample of each fusion protein was assessed by western blotting (fig. 4).

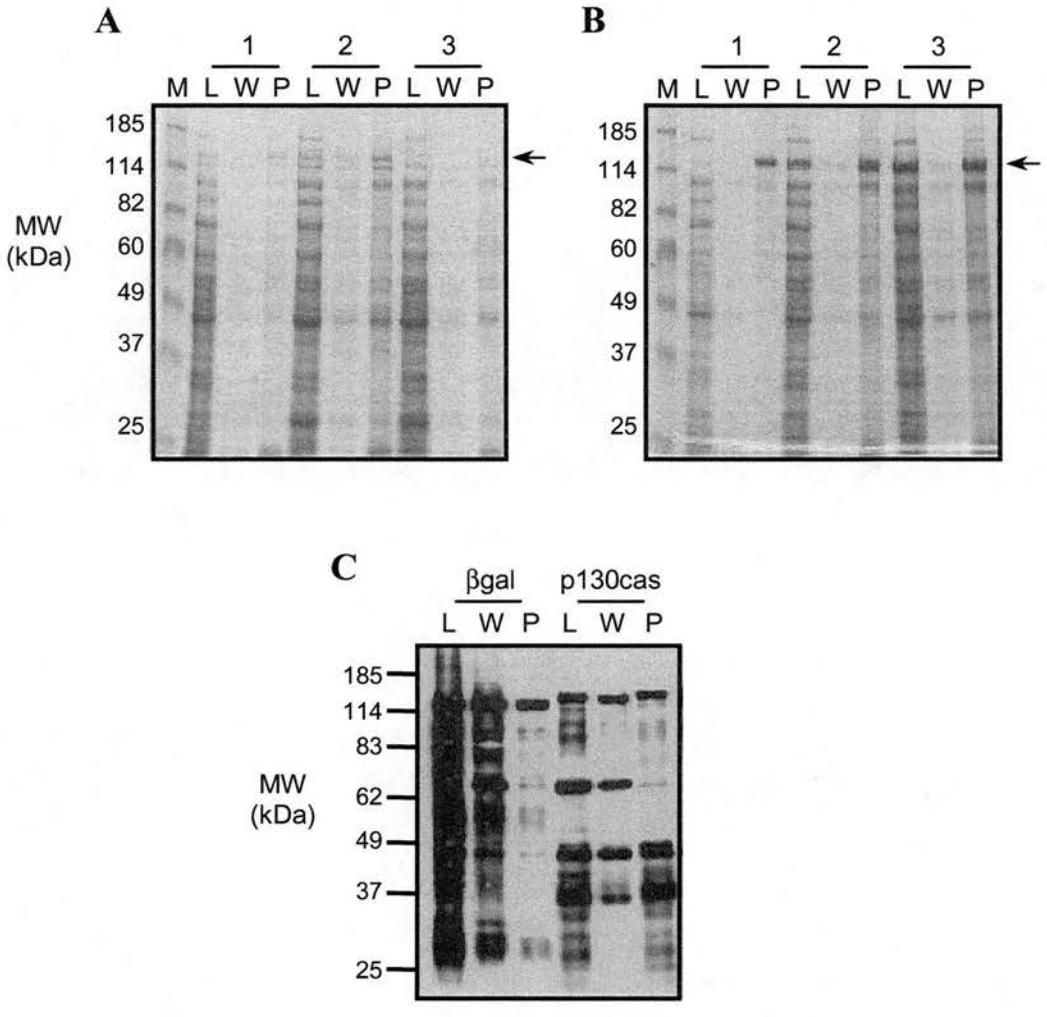

Figure 4. Identification of clones expressing high levels of TAT/p130cas and TAT/βgal fusion protein

E.coli strain BL-21 was transformed with pTAT/HA-p130cas and pTAT/HA-βgal constructs. Single colonies were amplified in liquid culture and expression of fusion constructs assessed by SDS-PAGE and Coomassie blue staining. High expressing clones were semi-purified by immunoprecipitation of proteins with Ni-NTA resin. **A** Coomasie blue staining of SDS-PAGE gel of p130cas purification. **B** βgalactosidase purification. **C** Western blot of two highly expressing clones probed with mAb specific for the HA epitope (1:250). Putative fusion proteins are indicated by arrows in panels (A) and (B), TAT/p130cas (135 kDa), TAT/βgal (125 kDa). M, molecular weight marker; L, whole cell lysate; W, wash; P, semi-purified fusion protein.

Bulk production of TAT-fusion proteins: His-Tag purification

Starter cultures of G_2 and C_2, clones highly expressing TAT/βgal, and TAT/p130cas fusions respectively were grown in liquid culture O/N and used to inoculate 500ml (G_2) and 1L (C_2) of LB-broth containing 100μg/ml ampicillin. Cultures were grown until an OD_{600} of 0.5 was obtained and then induced with IPTG for 5 hours. Cells were lysed in denaturing conditions with buffer containing 8M urea, pH 8.0, lysates clarified by centrifugation and incubated with Ni-NTA resin plus 10mM imadazole (to minimise non specific binding). After incubation, the resin/lysate mix was loaded into 50ml syringes containing a nylon wool plug (to prevent loss of resin through the syringe), and allowed to pass through by gravity flow, with the effect that the Ni-NTA beads sedimented with fluid flow, creating a resin column. The column was washed to remove non-specifically bound proteins with urea buffers at pH 8.0, and pH 6.3, and eluted in denaturing buffer in fractions at pH 5.9 and pH 4.5. Samples from each step of the column were analysed by SDS-PAGE and western blotting. Coomasie-blue staining of acrylamide gels revealed the presence of clean bands of ~135 kDa and 125 kDa for p130cas and β-gal fusions, respectively (fig. 5a,b). This corresponds to the addition of the TAT leader sequence of ~3.5 kDa, and the HA tag of ~1.5 kDa. Both proteins eluted at pH 5.9, corresponding to monomeric protein, but also at pH 4.5 that allows the elution of multimers and aggregates. Western blotting with anti-p130cas and anti-HA mAb demonstrated these bands corresponded specifically to p130cas and βgal (fig. 5c,d).

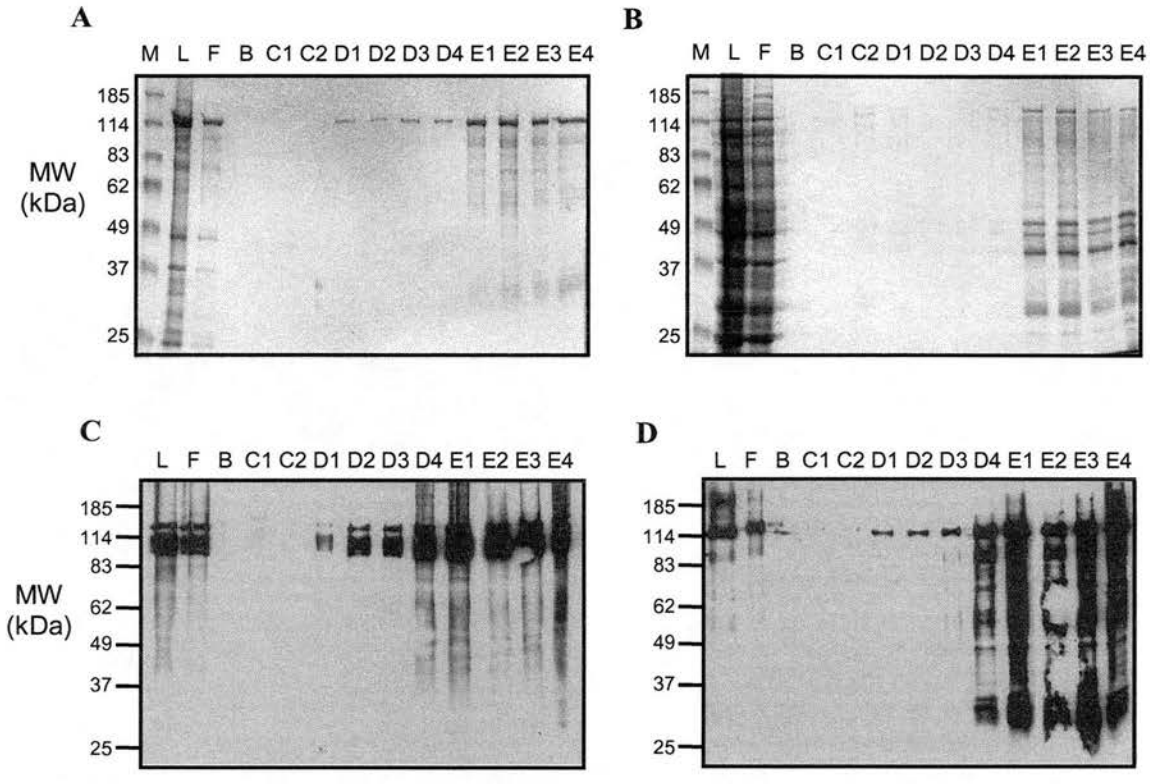

Figure 5. His-Tag purification of TAT/p130cas and TAT/βgal fusion proteins
Bulk cultures of high expressing clones induced with IPTG were lysed with 8M urea and purified over a Histidine binding Ni-NTA column to isolate tagged proteins TAT/p130cas (A, C) and TAT/βgal (B, D). Washes and elutions were assessed for protein content by SDS-PAGE and together with Coomassie blue staining (A, B) or western blotting with mAb specific for p130cas panel C (1:5000), or HA panel D (1:250). TAT/p130cas (135 kDa), TAT/βgal (125 kDa). M, molecular weight marker; L, crude lysate; F, column flow through; B and C, washes with buffers B and C; D and E, elution fractions with buffers D and E.

Shock refolding of fusion proteins on an ion-exchange column

Efficient transduction of TAT fusions requires linearisation of the protein in order for it to traverse the lipid membrane. Correctly folded proteins that are stable with a low ΔG are difficult to unfold and are hence poor transducing substrates (Nagahara et al., 1998). Proteins capable of highly efficient transduction are characterised as being unstable (high ΔG), misfolded and aqueous soluble. We can produce proteins in this state by shock refolding from urea to aqueous solution on an ion exchange column.

Pooled fractions of fusion proteins from the Ni-NTA column were diluted with HEPES buffer to a urea concentration of 4M, and applied to a ResourceQ column pre equilibrated with a low salt HEPES buffer, and protein eluted with 1M NaCl in twenty 1ml fractions. 2 μl of each fraction was spotted onto nitrocellulose membrane and probed with p130cas and HA mAb. The dot blots revealed elution in fraction 2 for both p130cas and βgal, with further elution at decreasing levels in the following 18 fractions (fig. 6a,b). Protein present in these fractions appeared at the correct molecular weight when subsequently analysed by SDS-PAGE and western blotting (fig. 6c,d). Fractions containing protein were therefore pooled and concentrated by centrifugal filtration, then applied to disposable PD-10 columns to remove salt and allow elution in PBS.

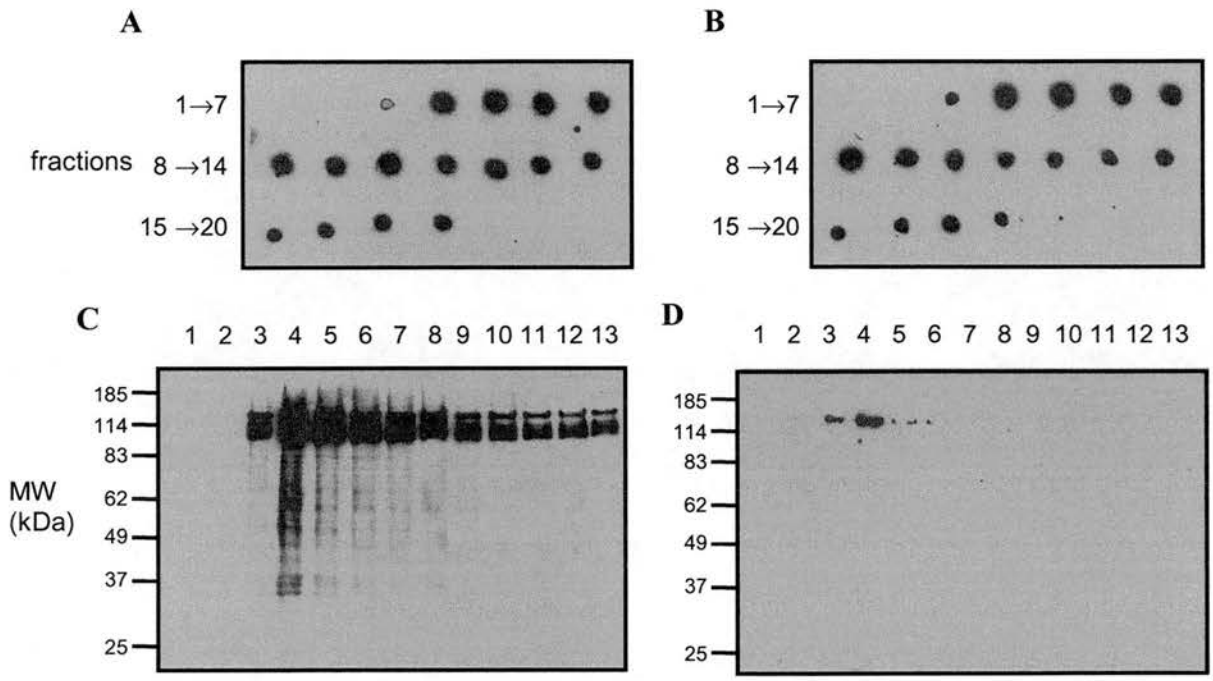

Figure 6. Purification of fusion proteins by gravity flow ionic exchange
Ni-NTA fractions containing fusion protein were pooled and passed through a gravity flow monoQ ion exchange column for shock refolding of TAT fusion proteins. Twenty 1ml fractions were collected and assessed for protein content by dot blot analysis (A, B) and fractions 1-14 by SDS-PAGE together with western blotting (C, D) using mAb specific for p130cas (A, C) or HA (B, D).

Transduction of TAT into monocyte/macrophages

Dowdy and colleagues have previously demonstrated transduction of proteins up to 110 kDa into a range of cell types. Before assessment of the potential biological functions of the TAT/p130cas fusion protein, we must first demonstrate its cellular transduction. Reports suggest that TAT fusions are able to transduce into cells within 15 min of incubation, and reach a steady state (as proteins can theoretically transduce back out) after 1hr (Nagahara et al., 1998). Other reports have suggested transduction within 15 min, but loss of biological function after 45 min (Alblas et al., 2001). In preliminary experiments we chose a 1hr time point, and assessed transduction of TAT/p130cas and TAT/βgal into DX-treated macrophages by SDS-PAGE and western blotting. Macrophage monolayers were washed once to remove serum, and incubated with TAT fusion proteins. After 1hr, supernatants were removed, and cells lysed. At this time point we could not detect transduction of the TAT/βgal fusion, however TAT/p130cas had appeared to enter the cell. Probing blots with a p130cas specific mAb revealed a protein of approximately 100 kDa, approximately 35 kDa smaller than the predicted TAT-HA/p130cas product, suggesting cleavage of the fusion protein. In addition to the 100 kDa band, there appeared to be extensive lower molecular weight degradation products visualised by the p130cas mAb. We therefore repeated the experiment with earlier time points of 15 and 45 min. Monocyte/macrophages matured for 5-days ± 1μM DX in 48 well plates were incubated with 100μl of TAT/p130cas for 15, 45 and 60 min, and 100μl of TAT/βgal for 15 and 60 min intervals. Supernatants were removed and retained and the cells washed briefly in PBS to remove bound, non transduced protein, before lysis with RIPA buffer. Analysis of lysates and supernatants by SDS-PAGE and western blotting with p130cas, and HA specific mAb, revealed TAT/p130cas fusion protein was able to enter macrophages within 15 min of incubation (fig7). Cellular transduction appeared to protect the protein from non-specific degradation occurring in the supernatant, however the major species in cell lysates was a band of approximately 100 kDa. A band of the correct molecular weight (135kDa) corresponding to the band pattern of whole protein was present in supernatants, suggesting that cleavage to the 100 kDa form may be a specific cellular event rather than non-specific proteolytic degradation. Alternatively the 100 kDa species present

in the supernatant also may transduce more efficiently into the cell, and would therefore be more concentrated in cell lysates. Transduction did not significantly increase with increased incubation times, nor was there an increase in cellular degradation of the p130cas fusion protein, in contrast to previous experiments. One possibility was that failure to rinse cells after removal of the supernatant, to prevent wash out of protein, may have left residue degradation products on the surface of macrophages. TAT/βgal could not be detected inside either control or DX macrophages after 15 or 60 min (fig7), however the fusion protein could be detected in the supernatants. A band of between 70-75 kDa was present in cell lysates from cells incubated with the TAT/βgal fusion. This may represent transduced protein that has been processed by the cell, or alternatively, a non-specific band. Full length transduced TAT/βgal may not be detected due to the reduced sensitivity of the HA mAb compared to the p130cas mAb.

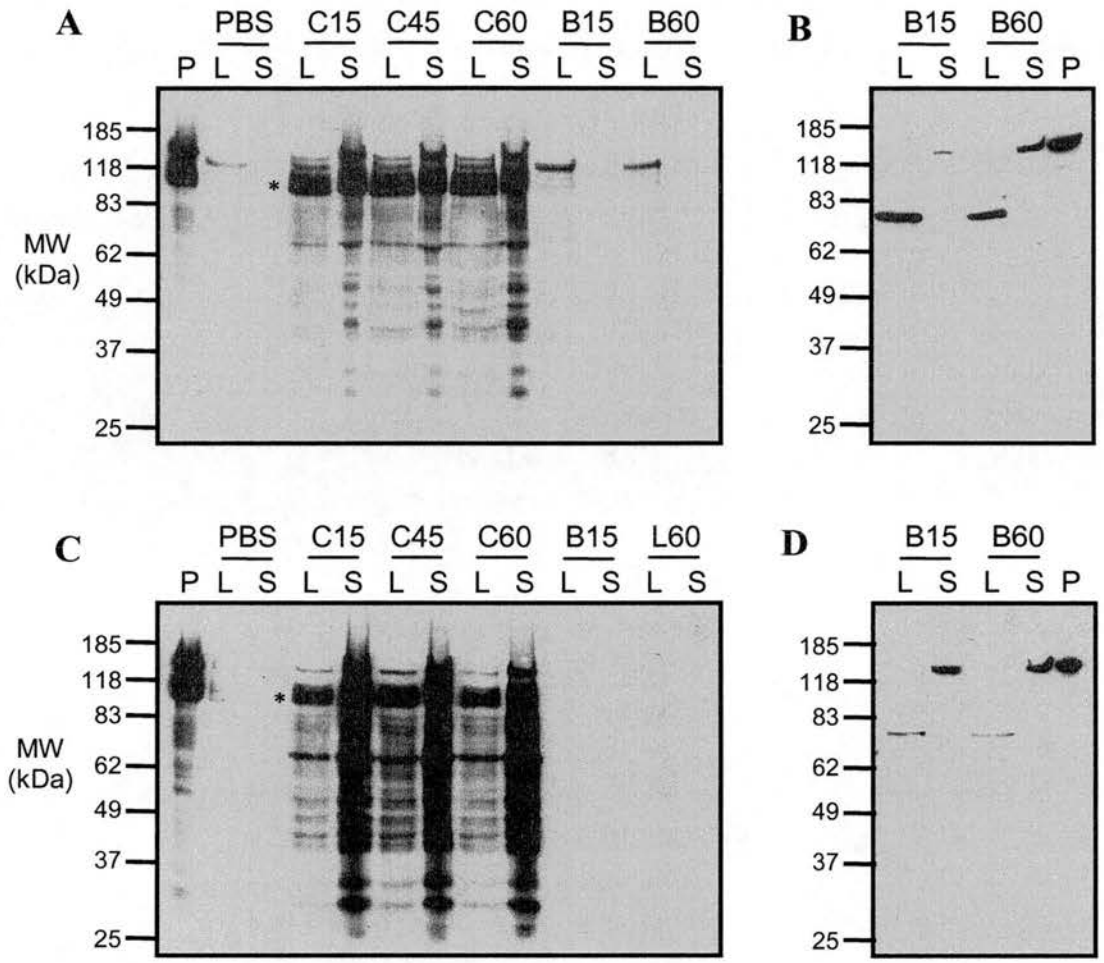

Figure 7. Transduction of TAT fusion proteins into macrophages

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days in 48 well plates. Cultures were incubated with 100μl of TAT/p130cas or TAT/βgal for 15-60 min prior to lysis and assessment of the level of protein transduction by SDS-PAGE and western blotting using mAb specific for p130cas: panels A and C, or HA: panels B and D, in either control non-DX-treated (A, B) or DX-treated (C, D) macrophages. Note the level of transduced p130cas compared to endogenous levels present lanes corresponding to control non-DX-treated macrophages treated with PBS/glycerol and TAT/βgal. C, TAT/p130cas; B, TAT/βgal; L, lysates; S, supernatants; P, purified protein control. The putative "100 kDa band" referred to in the text is marked *.

The effect of TAT fusion proteins on macrophage phagocytosis of apoptotic neutrophils

The aim of this work was to reintroduce p130cas back into DX-treated macrophages to test our hypothesis that p130cas was a negative regulator of phagocytosis. As cell loss can occur during the plate based phagocytosis assay we used flow cytometry to assess phagocytic uptake. Monocyte/macrophages were cultured in 48 well plates for 5 days in the presence or absence of 1μM DX. Cells were washed once to remove serum and incubated with 100μl of either PBS, PBS/glycerol, TAT/βgal or TAT/p130cas for 15min. Supernatants were removed and phagocytosis of apoptotic neutrophils assessed as previously described. Both untreated and DX-treated cells pre-incubated with PBS only showed average levels of phagocytosis of 7.5% and 48.9% (non-DX-treated and DX-treated respectively). However, incubation of macrophages in PBS/glycerol appeared inhibitory to phagocytosis. Levels of uptake in untreated cells were reduced to 4.1%, and in DX-treated cultures just 7.3% of macrophages were positive for engulfment of particles. In addition samples that had been incubated with PBS/glycerol appeared to contain fewer macrophages suggesting cell loss at some point during the assay. Similar findings were obtained for cultures treated with TAT/βgal. In DX-treated cultures phagocytosis remained at 7.2%, however phagocytosis by non-DX-treated macrophages appeared to be improved in the presence of protein (7.7%). Pre-incubation with TAT/p130cas demonstrated similar phagocytosis levels in untreated cells as for TAT/βGal (9.1%), but decreased phagocytosis compared to TAT/βgal in DX-treated cells (2.2%).

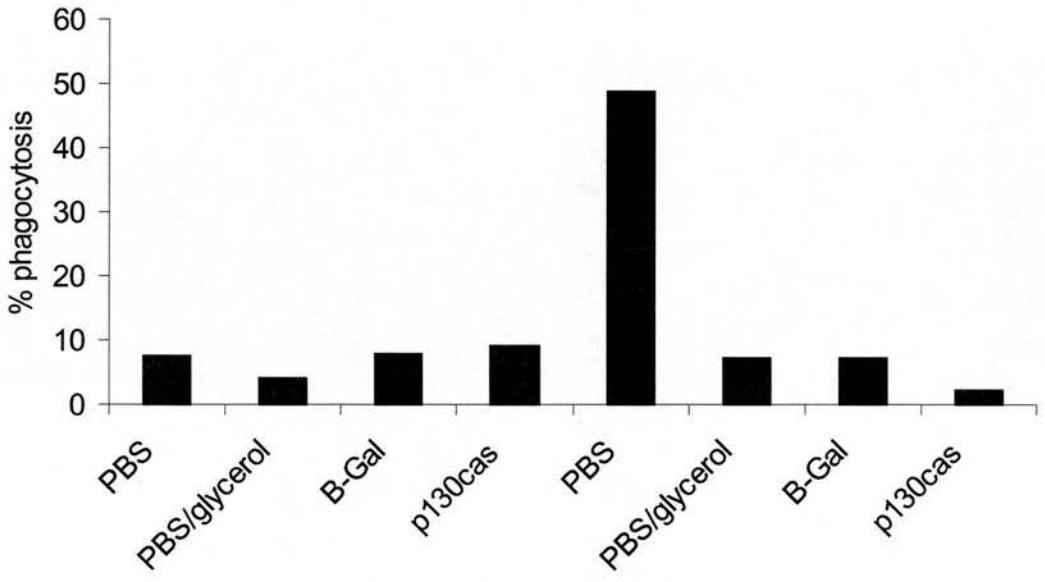

Figure 8. Effect of fusion proteins on phagocytosis of apoptotic cells
Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX for 5 days in 48 well plates. Cultures were incubated with 100μl of TAT/p130cas or TAT/βgal for 15 min, and macrophage phagocytic capacity for apoptotic neutrophils assessed by flow cytometry. Results show one representative experiment of two that were performed.

Discussion

Glucocorticoids reprogram monocyte to macrophage differentiation resulting in the alteration of a number of adhesion signalling pathways. In particular, DX down regulates p130cas expression. We have hypothesised that the decrease in protein level of p130cas contributes to the morphological changes induced by glucocorticoids and the augmentation of phagocytic capacity. To test our hypothesis we created a TAT/p130cas fusion protein for the direct transduction of p130cas into DX-treated macrophages. Initial experiments suggested that the p130cas fusion protein was able to enter DX-treated and untreated macrophages as assessed by western blotting of cell lysates. However the control TAT/βgal protein could only be detected in supernatants. This may be due to a complete failure of the protein to transduce, or a lack of sensitivity of the anti-HA mAb, which may not allow the visualisation of low levels of transduced protein. Despite transduction into the cell TAT/p130cas did not alter macrophage morphology as assessed by phase contrast microscopy (data not shown). An absence of effect in non-DX treated control macrophages could be due to the presence of endogenous p130cas. However, increased cell spreading, membrane ruffling and formation of stress fibres was observed in cell lines overexpressing p130cas and p130cas$^{-/-}$ fibroblasts re-expressing the protein after transfection using mammalian expression vectors (Cheresh et al., 1999; Eisenmann et al., 1999; Honda et al., 1998; Kiyokawa et al., 1998). Additionally although TAT/βgal may have been entering cells in these experiments at a low levels, it was unable to metabolise the chromogenic substance X-gal, raising questions as to the functionality of the fusion proteins produced. Sequencing of the pTAT-p130cas expression construct revealed four polymorphisms that had arisen during PCR amplification, resulting in four amino acid substitutions. Only one change Thr→Ala occurred in a functional domain of p130cas. This substitution was proximal to a putative tyrosine phosphorylation motif in the substrate domain. Whether this affects p130cas phosphorylation, or if the other substitutions alter protein folding is not know, but should be addressed if extensive future work is undertaken using this construct.

Stability of TAT fusion proteins

The lack of effect on macrophage morphology of p130cas fusion protein may be due to failure to reach levels comparable to endogenous expression, although western blot analysis suggests that p130cas transduced into control cells is in excess of normal basal levels. However, the p130cas species present is not the full-length protein. Endogenous p130cas in human macrophage lysates (see chapter 4, fig. 7) appears as a doublet of protein bands of 130kDa and 120kDa when analysed by SDS-PAGE and western blotting. The doublet is specific for human p130cas as it is not apparent when anti-p130cas mAbs are used to probe cell lysates generated from J774s , a mouse macrophage cell line. This may be due to the presence of a cryptic termination signal, specific cleavage sites, or alternative splicing of human p130cas mRNA. p130cas has been shown to be cleaved by caspases during apoptosis resulting in the production of a number of fragments ranging between 31 and 74 kDa (Kook et al., 2000). Over exposure of p130cas blots of macrophage whole cell lysates reveal that endogenous p130cas is susceptible to non-specific protein degradation, with the generation of a number of lower molecular weight bands (results not shown). Visualisation of purified p130cas fusion protein by SDS-PAGE and western blotting also demonstrates the presence of two major bands plus a number of degradation products. The upper major band approximately 135kDa is likely to correspond to p130cas plus the TAT/HA leader sequence, an extra 5kDa (although TAT proteins have been shown to migrate up 10kDa larger than their actual size (Nagahara et al., 1998). In western blot analysis the lower band at ~100kDa is too small to correspond with the lower 120 kDa doublet plus TAT leader. Additionally the stoichiometry of the two bands in p130cas fusion protein samples is different to endogenous p130cas. The two products of TAT/p130cas are present throughout purification, suggesting that either two species are produced in the bacterial expression system, or that the fusion protein is unstable and prone to proteolytic degradation from the outset. During initial purification experiments, protein from the Ni-NTA column was left in urea overnight before further purification on the monoQ column. Even in 8M urea, residual protease activity was able to cleave p130cas, to a protein of approximately the same size as the lower 100

kDa band. Transduction of proteins from 18-120 kDa have been reported by Dowdy and colleagues. Although inert particles up to 40nm have been reported to pass into cells using the HIV-TAT system (Lewin et al., 2000), there may be an upper size limit for the transduction of highly charged moieties such as proteins, as is seen with the PTD of Antennapedia (Derossi et al., 1998). The production of two species of p130cas, although not intended, may be fortuitous in allowing entry of the lower molecular weight protein, however size constraints may contribute to the lack of transduction of the TAT/βGal fusion.

Proteolytic degradation of the protein with the generation of lower molecular weight species occurred in a pattern similar to that seen with endogenous p130cas, but to a greater extent. The presence of degradation products was most pronounced in supernatant fractions, with a reduction in cleavage upon transduction inside the cell. Cytoplasmic p130cas appeared to be protected against non-specific cleavage and the generation of lower molecular weight products. In contrast the βgal fusion protein did not appear to be cleaved in the supernatant. This may be due to lower sensitivity of the anti-HA mAb, failing to detect TAT/βgal degradation products that would be present at lower concentrations. Addition of protease inhibitors during purification would inhibit cleavage, however interpretation of morphological and functional changes in the presence of protease inhibitors may not be possible. Incubation of monocyte/macrophages with fusion protein, or just the carrier, PBS/glycerol resulted in cell loss and a decrease in phagocytosis, suggesting glycerol may compromise macrophage viability and induce cleavage of p130cas by the induction of an apoptotic program. Cell viability could be assessed by either blotting the resulting lysates for cleavage of pro-caspase 3 or assessing levels of Annexin V binding in duplicate samples by flow cytometry during the eating assay. Dowdy and colleagues transduce proteins in the presence of BSA (Nagahara et al., 1998). Although we undertake our phagocytosis assays in the absence of serum due to its capacity to opsonize apoptotic cells, the use of heat-inactivated BSA may be a possible means to stabilise both protein and cells.

p130cas contains a number of protein/protein interaction domains required for activation of its downstream signalling pathways, protein cleavage with the loss of specific motifs may therefore compromise function. N-terminal degradation would remove the TAT leader sequence preventing transduction into the cell, although specific cleavage of the leader sequence once inside the macrophage may still occur. It is more likely therefore that degradation occurs from the C-terminal end of the protein. The C-terminal region of p130cas contains a potential hetero/homodimerisation motif, plus a signature motif for tyrosine phosphorylation by src family kinases. p130cas is a major mediator of src-signalling effecting changes in cell morphology, adhesion and migration. Disruption of this interaction may prevent the observation of morphological changes following fusion protein transduction.

In addition to detection of transduced proteins by western blotting, we attempted to visualise TAT/p130cas and TAT/βgal inside macrophages using immunofluorescent staining, to enable assessment of localisation patterns, and provide evidence that the protein was correctly folded and functional. However both p130cas and HA antibodies gave a large amount of background immunofluorescence in cytospin preparations of transduced cells. This is likely to reflect the binding properties of the specific monoclonal and polyclonal antibodies used. Additionally, the lower molecular weight degradation products that are faintly apparent in cell lysates may also contribute to an apparent non-specific staining, therefore the issue of protein cleavage may need to be addressed before immunofluorescent staining is possible.

Another potential problem with protein transduction, is that in addition to cleavage compromising fusion protein function, re-folding of both p130cas and βgal after transduction may not be correct. Improperly folded proteins would be targeted to the proteosome, and rapidly degraded. However, as we still detect p130cas and βgal after 1hr of incubation this is unlikely to occur. Folding of large complex molecules may be further compromised when transducing proteins between species. The bacterial strain BL-21 expressed βgal and was able to metabolise X-gal producing blue pigmentation, suggesting functionality of the protein, however correct folding in

mammalian cells of this bacterial protein may be limited. Furthermore the copy number of correctly folded proteins may be too low for the visualisation of X-gal metabolism by microscopy.

We purified fusion proteins from the insoluble denatured phase to promote maximal recovery and production of "transducible" protein. However it has recently been shown that some fusions with TAT require purification from the soluble or aqueous fraction to become efficiently refolded in vivo. For example TAT fusion with *herpes simplex* virus thymidine kinase requires purification from the soluble phase. Unfortunately it has also been reported that βgal can only form the enzymatically active homotetramer when purified from 0 and 2M urea, but not 8M. Complete denaturation leaves the TAT-fusion in a highly transducible state, but with a poor rate of folding. Although loss of βgal activity does not enable us to graphically demonstrate protein transduction, its presence inside the cell still functions as a control for non-specific effects generated by the transduction of a protein in excess of 100kDa into monocyte/macrophages. Further production of TAT fusion proteins for studies in macrophages will require careful tailoring of purification procedures for each individual protein in order to obtain maximal function.

Although reconstitution of p130cas itself may not be sufficient to change the gross appearance of DX-treated cells, we may be able to detect subtle changes. Immunofluorescent staining of actin organisation or the re-appearance of podosome structures could potentially reveal functional effects of p130cas that are compromised in DX-treated macrophages. Furthermore, assessment of Rac activity, and pyk2 and paxillin phosphorylation after protein transduction would allow investigation of the specific signalling pathways altered by the decrease in p130cas expression. In the previous chapter we described a crude migration assay, in which DX-treated cells were able to migrate despite the absence of p130cas and active ERK. It would be very interesting to assess macrophage migration after reconstitution with p130cas.

Effect of TAT fusions on phagocytic capacity

In the functional studies, we found that pre-incubation with PBS/glycerol disrupted phagocytosis and induced a slight cell loss in control macrophages and a marked reduction of cell number and phagocytosis in DX-treated cultures. The viscosity of glycerol may prevent settling of neutrophils onto the macrophage monolayer. In the first preliminary experiment apoptotic cells were added on top of glycerol/PBS or fusion protein, to limit potential transduction of fusion protein back out of the cell when the extracellular concentration of protein was reduced by the addition of media containing apoptotic cells. This could account for the decrease in phagocytosis, but not the dramatic cell loss observed in DX-treated cultures. Furthermore, similar results were obtained when glycerol/PBS was removed before application of the apoptotic meal in subsequent experiments. Alternatively the viscosity of the glycerol mix may itself compromise cell adhesion. Alternatively glycerol, even at 10-15% may be toxic to monocyte/macrophages. Decreased viability would inhibit phagocytosis as well as causing detachment of cells. Possible apoptotic effects could be assessed by examination of nuclear morphology by cytospin analysis and assessment of phosphatidylserine externalisation with AnnexinV binding. Phagocytosis rates were improved slightly in the presence of the TAT/βgal fusion protein, compared to PBS/glycerol, and effect that is unlikely to be specific. Dowdy and colleagues transduce proteins in the presence of BSA (Nagahara et al., 1998), the use of heat inactivated serum may promote adhesion and viability, and prevent possible opsonization of apoptotic particles with complement proteins, that might complicate interpretation of phagocytosis data.

An alternative approach to the use of glycerol would be to freeze protein in a different carrier such as sucrose, or to obtain concentrated preparations of fusion protein that would allow dilution of glycerol when the fusion protein is used in functional studies. This would require optimisation of protein production and purification methods. The intracellular location of p130cas suggests that post-translational modifications such as glycosylation are not required permitting use of bacterial expression systems that allow higher yields. Protein solubility can be compromised if the protein is toxic or expressed in large quantities, resulting in

sequestration into insoluble inclusion bodies within bacteria. Protein can be accessed by sonication in the presence of a denaturant such as 8M urea or 6M guanidine hydrochloride. Although we were able to recover considerable amount of protein from the bacterial cytoplasm the addition of a sonication step to our purification procedure may further increase the total yield. Very long recombinant proteins can also undergo premature termination during translation in bacteria due to differences in codon usage between prokaryote and mammalian systems. Attachment of the His-tag to the C-terminus of the protein would select for full-length proteins during purification. The lower molecular weight band observed in purified TAT/p130cas could represent a terminal truncation during translation, data presented in figure 7 suggests it is more likely to be the result of proteolytic degradation. Unlike many smaller fusion proteins that are degraded by bacterial proteases, the length of both p130cas and βgal fusions preclude them from undergoing cleavage in the bacterial cytoplasm. Use of different protease inhibitors would limit proteolysis following lysis and inhibitors would not co-purify with fusion proteins on subsequent affinity columns, preventing contamination of the final protein solution.

In this chapter I have explored techniques for the transduction of proteins into macrophages. Further refinement of this technique would allow a more detailed analysis of the effects of p130cas expression on macrophage programming. For example manipulation of the p130cas protein by deletion of protein domains to generate dominant negative mutants, or constitutively active protein may also allow the elucidation of specific signalling pathways mediated by p130cas in normal macrophages. Most importantly use of this technology for the introduction of protein into monocyte derived macrophages will provide opportunities for the study of other key molecules involved in macrophage adhesion, differentiation and function.

CHAPTER 7: THE EFECT OF THE CYTOKINE ENVIRONMENT ON GLUCOCORTICOID "PROGRAMMING" OF MONOCYTE-MACROPHAGE DIFFERENTIATION

Introduction

The inflammatory response provides a defence mechanism against bacterial infection, injury and allergy. Recruitment of inflammatory cells and the release of inflammatory mediators is required for the elimination of the causative agent, however these processes must be tightly controlled to prevent escalation of the inflammatory reaction, and actively diminished to allow healing. Cells that mediate initiation of inflammation also act during the healing or resolution phase. Macrophages play a central role in innate immunity and the initiation of adaptive immune responses. Tissue injury is associated with an increased number of macrophages from the infiltration of circulating peripheral blood monocytes which differentiate into macrophages (van Furth, 1989). It has also been proposed that tissue macrophage numbers increase by local cell division (Sawyer et al., 1982). The role macrophages play during an immune response is dependent on the cytokine microenvironment which can be influenced by T_H1 or T_H2 lymphocytes (Jankovic et al., 2001; Mosmann and Sad, 1996). Infection with *Leishmania major* is the prototypical model of T_H1/T_H2 responses (for reviews see Alexander et al., 1999; Louis et al., 1998). T lymphocytes from resistant C57BL/6 mice produce a T_H1 cytokine, interferon-gamma (IFNγ), in response to the infection, resulting in macrophage activation, production of NO, and parasite killing. In contrast, T lymphocytes from susceptible BALB/c mice produce IL-4 associated with T_H2 responses that suppresses macrophage microbicidal function, and the infection persists. A large number of cytokines and growth factors have been shown to modulate macrophage function and "activation state", these arise from both activated T cells and also via autocrine effects from the macrophage itself. IFNγ, LPS and TNFα have been shown to be important mediators of classical macrophage activation, associated with the induction of pro-inflammatory responses and the

propagation of T_H1 cytokine production (Goerdt et al., 1999; Mills et al., 2000; North, 1978). In addition, other cytokines such as IL-2, IL-12 and IL-15 have been shown to promote macrophage pro-inflammatory actions (Munder et al., 1998b; Nathan, 1987). In contrast, cytokines such as IL-4, IL-10, IL-13, and glucocorticoid hormones have been shown to downregulate these responses (Becker and Daniel, 1990; Bogdan and Nathan, 1993; Kourilsky and Truffa-Bachi, 2001). Downregulation of pro-inflammatory functions was initially thought to be due to a "deactivation" of macrophage function. However, it has become apparent that deactivated macrophages are able to actively influence the inflammatory outcome via the production of T_H2 cells and cytokines. The induction of this phenotype has been termed "alternative activation" (Stein et al., 1992). Furthermore, there is evidence that cytokine release from macrophages and dendritic cells can act in a paracrine fashion on T lymphocytes promoting their polarisation into T_H1 or T_H2 cells, as well as influencing macrophage or dendritic cell functions. Thus, the T_H1/T_H2 paradigm has been expanded to encompass macrophages and dendritic cells with the resulting classification of M-1/M-2 (Goerdt et al., 1999; Mills et al., 2000), and DC1 and DC2 cell subtypes (Rissoan et al., 1999; Sallusto et al., 1999). M-1 cells are characterised by upregulation of immunoglobulin receptors, to promote uptake of opsonzied particles, and increased NO production from an upregulation of expression of inducible nitric oxide synthetase promoting the destruction of ingested pathogens (Munder et al., 1998a; Munder et al., 1999). In contrast M-2 or alternatively activated macrophages express receptors for pathogen associated molecular patterns such as the macrophage mannose receptor (Stein et al., 1992), the β-glucan receptor (Mosser and Handman, 1992), scavenger receptor type I (Geng and Hansson, 1992), and CD163, a member of the cysteine rich family of scavenger receptors (Hogger et al., 1998). Recognition of pathogens by these pattern recognition receptors induces the production of pro-inflammatory cytokines (Garner et al., 1994), further emphasising that this macrophage phenotype is not de-activated. Despite an increased capacity for phagocytosis of pathogens, M-2 macrophages show decreased production of O_2, and NO upon stimulation (Becker and Daniel, 1990; Munder et al., 1998a; Munder et al., 1999), but express enhanced levels of MHC class II molecules such as HLA-DR and HLA-DQ, suggesting increased antigen presentation (Becker

and Daniel, 1990). Decreased NO production is thought to result from a switch in metabolism of arginine to ornithine from the induction of expression of arginase (Munder et al., 1999). Ornithine, as a precursor for polyamines, promotes cell replication, facilitating cell renewal during wound healing (Williams-Ashman and Canellakis, 1979). In contrast, NO inhibits proliferation and induces cell death (Nathan and Hibbs, 1991). *In vivo*, alternatively activated macrophages are found in normal healthy placenta and lung, during the healing phase of acute inflammatory reactions, in chronic inflammatory disease states such as rheumatoid arthritis and psoriasis, during wound healing and angiogenesis (Djemadji-Oudjiel et al., 1996; Gratchev et al., 2001; Kodelja and Goerdt, 1994; Kodelja et al., 1997; Mues et al., 1989; Szekanecz et al., 1994). Interestingly, macrophage phagocytosis of apoptotic cells has been suggested to promote alternative activation in the context that uptake of apoptotic particles downregulate the secretion of inflammatory mediators, and promote the release of anti-inflammatory cytokines such as TGFβ (Fadok et al., 1998). The suppressive function of these macrophages suggests they may be more akin to a T_H3 phenotype characterised by secretion of TGFβ (Chen et al., 1994). The capacity for phagocytosis by M-2 macrophages has not been extensively studied, however the effect of T_H1 type cytokines on uptake has produced equivocal results (Erwig et al., 1998; Ren and Savill, 1995). It is worth remembering that the T_H1/T_H2 paradigm represents two extremes of a spectrum of possible outcomes, and although it is possible to produce T_H1 or T_H2 responses in animal models, the real *in vivo* situation may represent a mid-point between these two extremes. Similarly the phenotype of normal tissue macrophage populations is likely to be between macrophage M-1 and M-2 states.

IFNγ, thought to induce an M-1 phenotype, induces the formation of multinuclear giant cells (MNGCs) (Fais et al., 1994; Most et al., 1990; Weinberg et al., 1985). *In vivo*, MNGCs are found in granulomas during chronic inflammatory disease such as infection with *Mycobacterium tuberculosi*, sarcoidosis, Crohn's, and primary bilary cirrhosis (for review see Fais et al., 1997). They consist of macrophages and MNGCs centered around an infectious focus, with lymphocytes aggregating around the cirumference, providing a possible source of fusion-inducing IFNγ. Generation

of multinucleated cell granulomas is dependent on the recruitment of extravasated monocytes, as tissue macrophages appear refractory to IFNγ induced fusion (Most et al., 1997). Erwig et al., (1998) have recently shown that *in vitro* macrophage programming is dependent on the first stimuli encountered. Monocytes infiltrating to the inflammatory site at different periods of the immune response may therefore be pre-programmed to undertake specific functions. We have demonstrated that glucocorticoids program monocyte/macrophage function and morphology when monocytes are exposed to DX early during maturation. Maturation of monocyte derived macrophages in culture induces the formation of a number of phenotypes as described in chapter 4. These include small "rounded" cells, larger spread cells, dendritic like migratory phenotypes, and multinucleated giant cells (MNGCs). The proportion of cell types, especially the number of giant cells present within a population differs between donors. This may be due either to soluble factors present in the autologous serum used to culture the cells, or a pre-programmed disposition for the formation of giant cells. In contrast, maturation of peripheral blood monocytes in the presence of DX prevents the formation of MNGCs, producing a culture of uniformily small, rounded macrophages, no matter how multinucleated the phenotype of the untreated counterparts. The formation of MNGCs can also be induced by IL-13 and IL-4 (DeFife et al., 1997; McNally et al., 1996), important mediators of the M-2/T_H2 paradigm. The production of a macrophage phenotype refractory to cytokine influence through DX-programming may prove detrimental *in vivo*. Alternatively cytokine inhibition of the DX-phagocytic phenotype, would have huge implications for the efficacy of glucocorticoids treatment for the resolution of certain inflammatory conditions. We therefore assessed the effect of the T_H1 and T_H2 cytokines INFγ and IL-4 on glucocorticoid maturation of monocyte/macrophage morphology, phenotype, and phagocytic capacity.

Results

DX inhibits giant cell formation

In order to assess the effect of cytokines on macrophage morphology induced by DX, peripheral blood monocytes were matured by adherent culture in 48 well plates for 5 days ±1μM DX in addition to 10ng/ml IL-4 or 10ng/ml IFNγ, and examined using phase contrast microscopy (fig.1). Maturation of monocytes in the presence of IFNγ induced a striking formation of MNGCs (fig. 1e), with cells up to 100μM in diameter. In contrast IFNγ/DX matured cultures demonstrated a uniform phenotype of small rounded cells devoid of multinuclear cells of any size (fig. 1f). In fact the IFNγ/DX treated cells appeared smaller and more rounder than those treated with DX alone (fig. 1b). It was noted that macrophages from IFNγ/DX cultures tended to exhibit increased detachment from culture plastic during cell washing, suggesting that changes in adhesion induced by DX were amplified by the presence of IFNγ. Although IL-4 has been described to induce the generation of multinucleated giant cells from peripheral blood monocytes in culture, we observed a dendritic cell-like phenotype. Furthermore, after 3-4 days maturation, cultures incubated with IL-4 demonstrated a clustering of cells reminiscent of cell aggregation during the formation of immature dendritic cells from peripheral blood monocytes in response to GM-CSF and IL-4. Autologous serum that is used to culture monocyte/macrophages is likely to contain GM-CSF, which may account for the differences in IL-4 induced morphology that we observe. The morphological changes during DX-programming were also dominant over the IL-4 induced phenotype. DX prevented clustering of cells and the resultant polarized morphology after 5 days maturation, instead producing smaller rounded cells. However the phenotype induced by maturation in the presence of IL-4 and DX was not as extreme as observed in DX only cultures. DX/IL-4 macrophages appeared slightly more irregular in shape with an increased number of cellular processes. Preliminary evidence suggested that DX-programmed cells were also refractory to the induction of giant cell formation by ligation of CD98 with the monoclonal antibody 4F2, preventing both clustering and fusion of cells (results not shown). No significant changes in 4F2 expression were observed after DX treatment suggesting changes in

intracellular signalling may inhibit fusion by this mechanism. The changes in morphology observed after cytokine-treatment was reflected in altered forward and side scatter properties of each subset of cells to a certain extent (fig.2). IL-4 treatment did not alter scatter properties compared to control, however the increased homegeneity observed with DX-treatment was maintained in DX/IL-4 treated cultures. Interestingly, although treatment with IFNγ induces formation of a heterogenous population of MNGCs, this was not reflected in the scatter profile, which appeared more homogenous than untreated cultures. The scatter profile of DX/IFNγ treated macrophages reflected the adherent morphology of these cells, showing a compact population of small homogenous cells.

A characteristic feature of giant cells and polarized/dendritic-like cells, as obtained by incubation with IFNγ and IL-4 respectively, is the extensive formation of podosome structures. In MNGCs podosomes are located in a cortical ring formation around the periphery of the cell. In osteoclasts, a cell of the monocyte lineage, the ring structure constitutes the "sealing zone" where resorption of matrix material occurs. In polarised or migratory cells, podosomes are formed at the leading edge of the cell, and are thought to contribute to the maintenance of cell polarity (see results chapter 3). Peripheral blood monocytes were matured by adherent culture on glass coverslips for 5 days as previously described, in the presence or absence of 1μM DX, 10ng/ml IL-4 or 10ng/ml IFNγ. Immunofluorescent staining of actin with rhodamine-coupled phalloidin in IFNγ treated cells, demonstrated the formation of punctate podosomal structures in a distinct pattern running around the periphery of the cell (fig.3). In IL-4 treated cultures, podosomes appeared both in the main body of the cell, and at the leading edge. Examples of cortical ring formation and cell polarisation were present in control cell cultures but to a lesser extent than in the cytokine treated populations. In contrast, the presence of DX completely inhibited the formation of podosmes in response to IFNγ and resulted in decreased actin organisation, similar to that observed in cells treated with DX only. However, in DX/IL-4 treated cultures although cells appeared small and rounded by phase contrast microscopy immunofluorescence revealed a more organised actin cytoskeleton with some evidence of adhesive contacts.

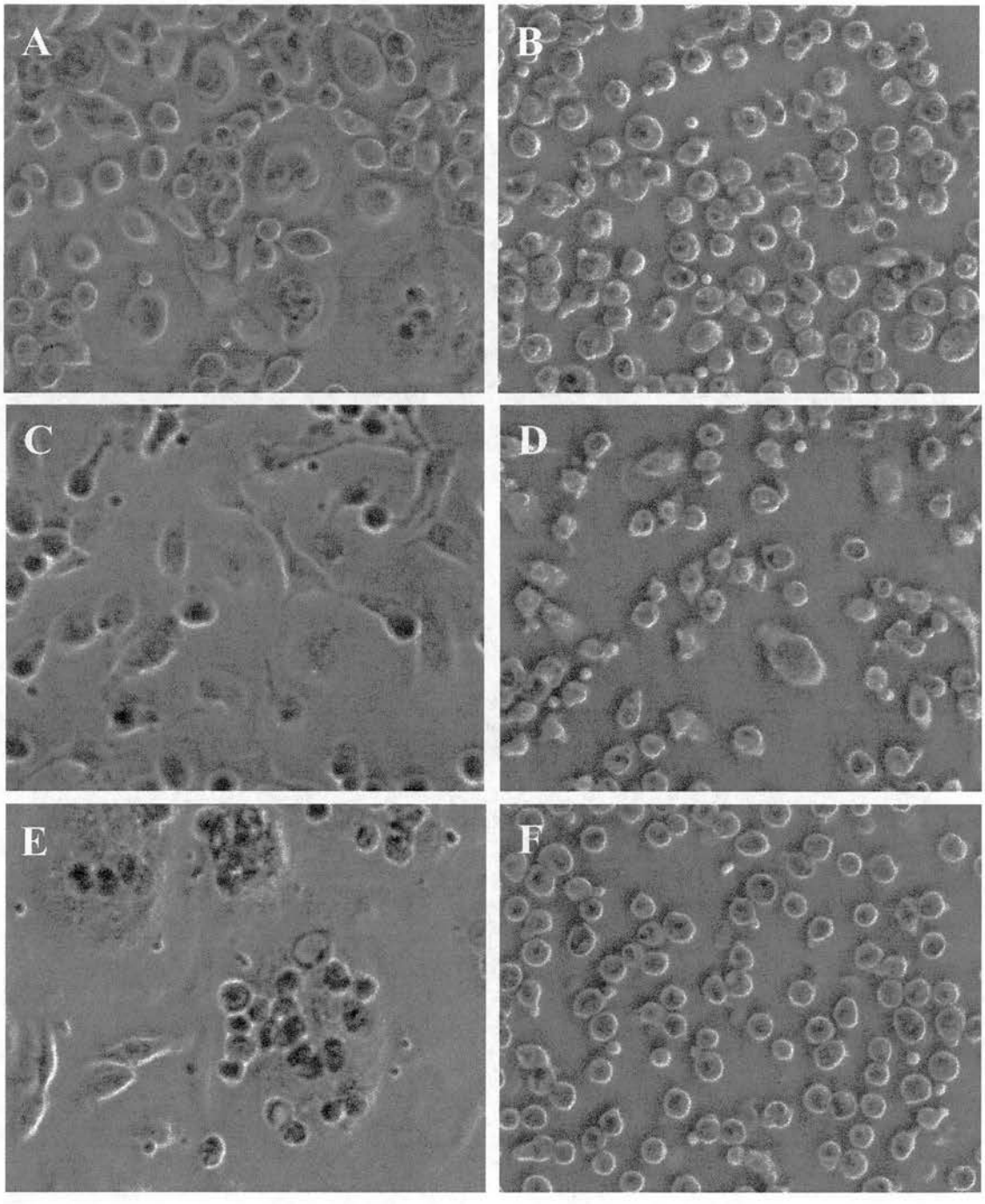

Figure 1. Effect of cytokine programming on macrophage morphology
Adherent peripheral blood monocytes were cultured for 5 days in the presence or absence of 1μM DX, 10ng/ml IFNγ or 10ng/ml IL-4 on tissue culture treated plastic. Morphology was assessed by phase contrast microscopy (x40 objective). **A** control untreated. **B** DX. **C** IL-4. **D** IL-4 plus DX. **E** IFNγ. **F** IL-4 plus DX.

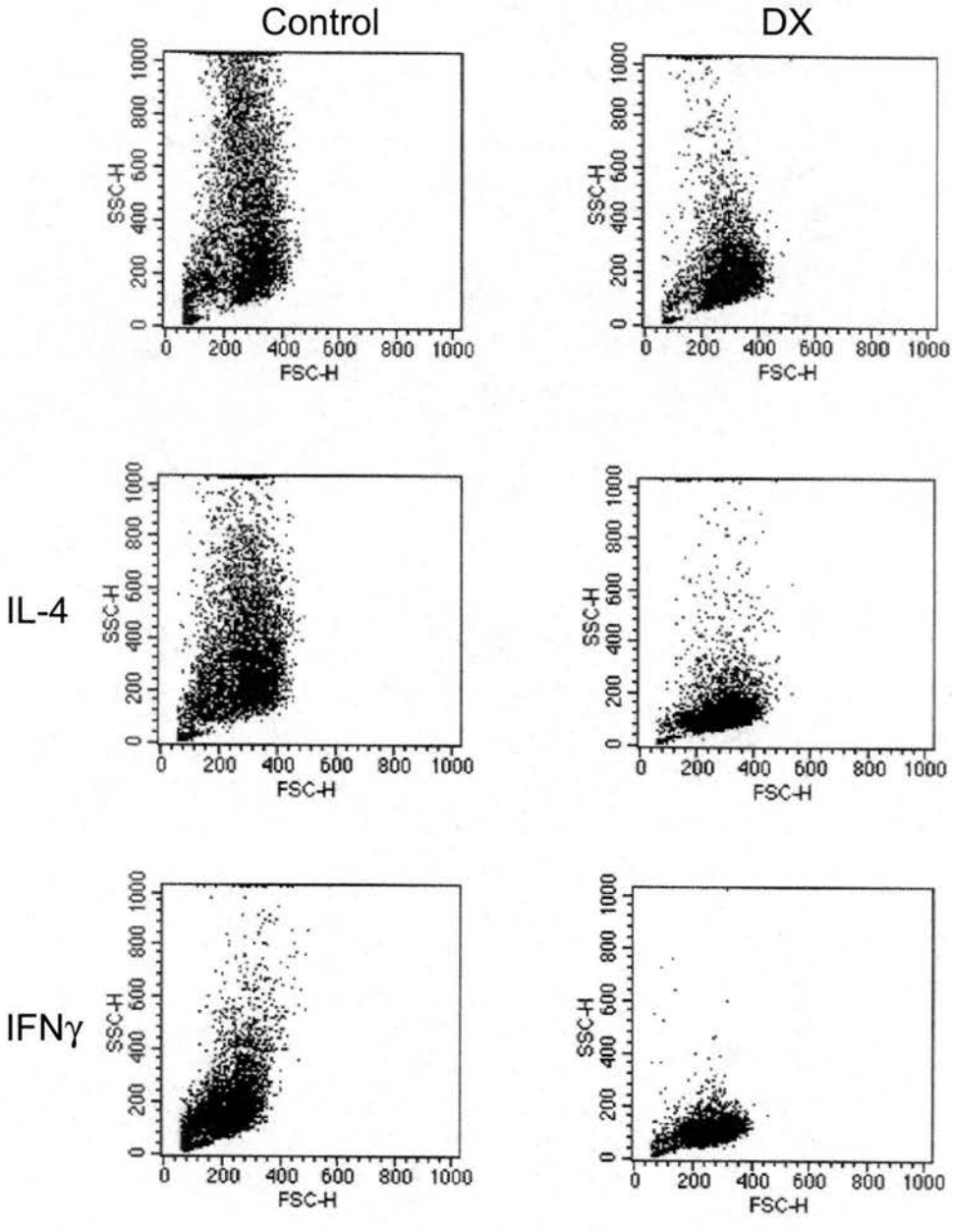

Figure 2. Increased homogeneity of macrophage morphology induced by cytokine programming

Adherent peripheral blood monocytes cultured on tissue culture treated plastic for 5 days in the presence or absence of 1μM DX, 10ng/ml IL-4 or 10ng/ml IFNγ. Cells were detached by incubation with on EDTA ice and laser scatter qualities assessed by flow cytometry.

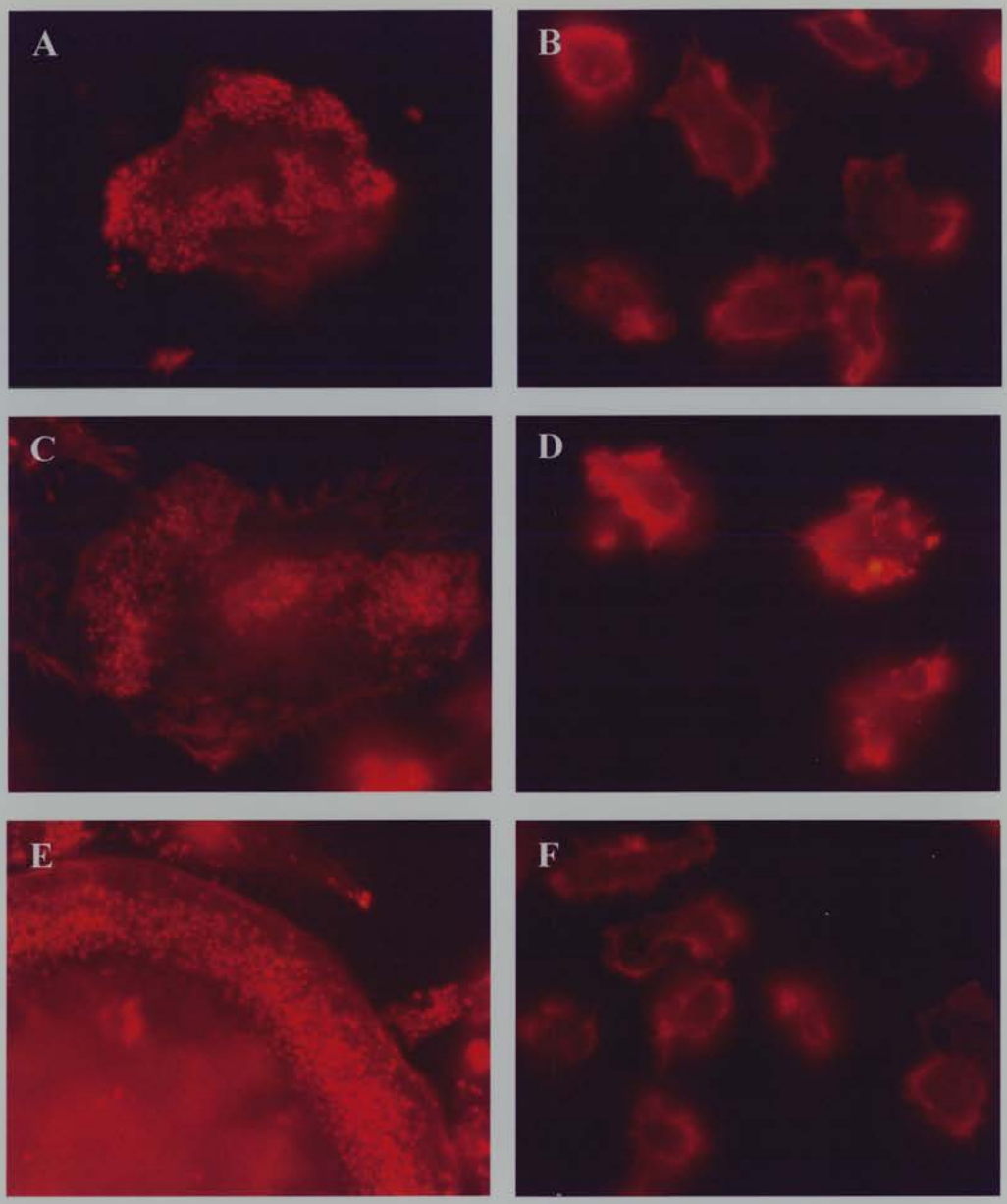

Figure 3. Effect of cytokine programming on actin organisation
Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1μM DX, 10ng/ml IFNγ or 10ng/ml IL-4. Localisation of actin was determined using rhodamine phalloidin together with fluorescence microscopy, x40 objective. **A** Media only, **B** DX, **C** IL-4, **D** DX/IL-4, **E** IFNγ, **F** DX/IFNγ. These representative micrographs illustrate typical punctate actin staining of "contact sites" and podosomes present in control, IFNγ, IL-4 and DX/IL-4 treated macrophages that are absent in DX-treated, and DX/IFNγ treated macrophages.

Cytokine influence on DX-programming of adhesion signalling

Morphological changes induced by DX correlate strongly with the down regulation of p130cas, an important regulator of actin cytoskeletal organisation and podosome formation (Honda et al., 1998; Nakamura et al., 1998), and the decrease in ERK activity, thought to be required for directed migration (Cheresh et al., 1999; Klemke et al., 1998). In order to see if DX programming was dominant over cytokine effects at a signalling level, we assessed the status of these two indices of DX maturation (detailed in chapters 4 and 5) in cells exposed to the cytokines IFNγ and IL-4. Monocyte/macrophages were matured for 5 days in the presence or absence of DX plus 10ng/ml IFNγ or 10ng/ml IL-4. Whole cell lysates were assessed for the presence of p130cas expression, or phosphorylated (i.e. active) ERK, by SDS-PAGE and western blotting (fig.4). Expression of p130cas and phosphorylation of ERK were decreased in DX-treated cultures compared to control. Treatment of monocyte/macrophages with IL-4 had no effect on levels of active ERK compared to untreated control, but caused a small increase in p130cas expression (fig. 4). IL-4 inhibted the effect of DX on ERK activity and p130cas expression, lysates from DX/IL-4 treated cultures contained both p130cas and active ERK. In contrast, IFNγ treated cells, although morphologically very different from DX-treated macrophages showed decreased levels of p130cas expression (in some experiments protein levels were below that of DX-treated macrophages), and a reduction in phosphorylated ERK. Downregulation in p130cas expression and ERK activity was also observed in DX/IFNγ-treated cultures.

Previous results have demonstrated that DX does not effect the activation of ERK directly, but via changes in upstream signals. To assess whether changes in ERK activity with IFN were due to a direct action of cytokine signalling on kinase function, the six subsets of cells were exposed to LPS to induce ERK activity, as described in chapter 5. LPS induced ERK phosphorylation in DX treated macrophages, and increased basal levels in control untreated cells as previously described. ERK activity was also induced in IFNγ and DX/IFNγ cultures, and basal levels of ERK activity were augmented in IL-4 and DX/IL-4, suggesting cytokines had no effect on the activation of kinase function (fig.5).

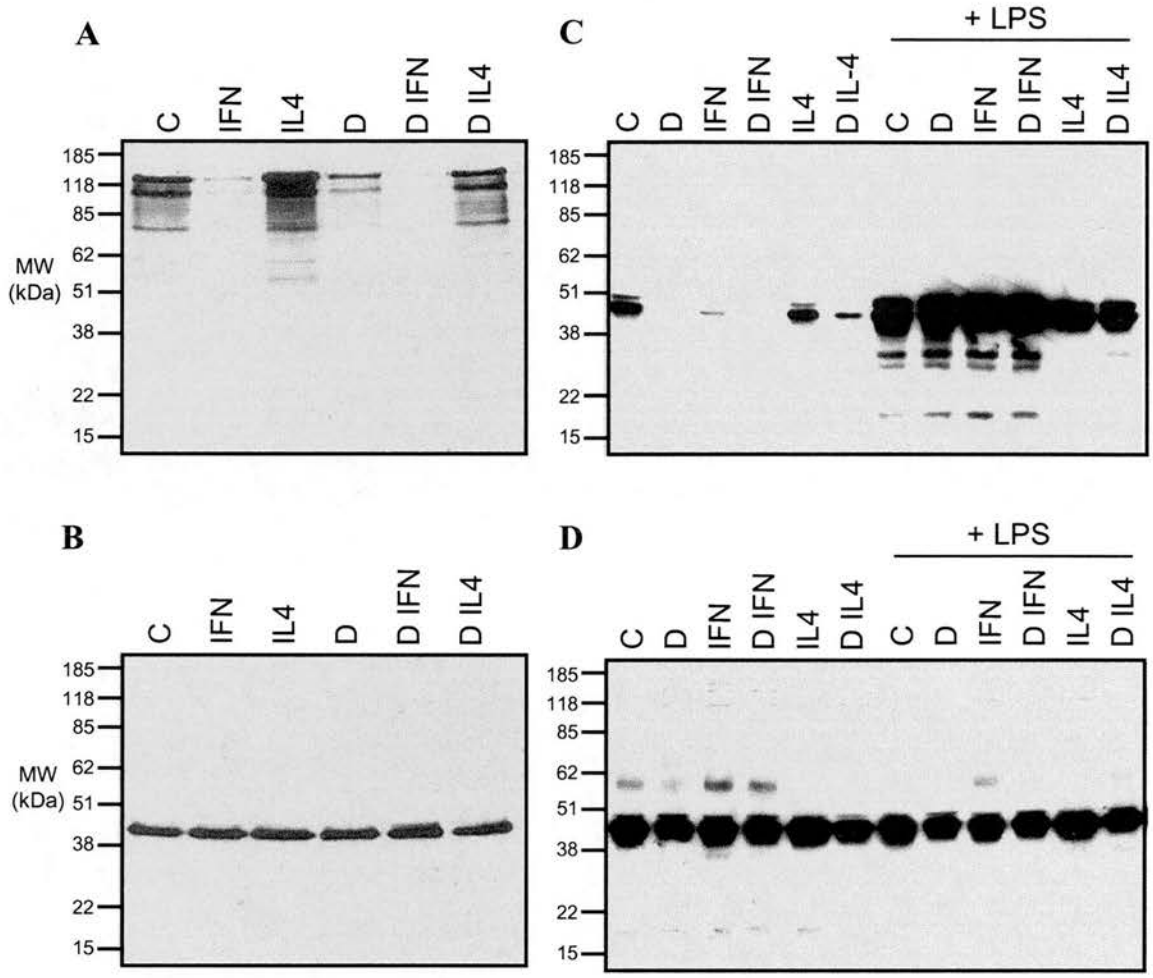

Figure 4. Effect of cytokines on expression of the adaptor protein p130cas and activity of the MAPK ERK.

Adherent peripheral blood monocytes were cultured for 5 days in the presence or absence of 1μm DX, 10ng/ml IFNγ, or 10ng/ml IL-4, and the influence of cytokines on macrophage reprogramming assessed by analysis of p130cas expression and ERK activity in whole cell lysates by SDS-PAGE and Western blotting. **A** Expression of p130cas (130 kDa). **B** CrkL (36 kDa). **C** and **D** Macrophages lysates were examined for the presence of phophorylated-ERK1/2 (active) (44/42 kDa) (C) and total ERK2 (42 kDa) (D), prior to and after incubation with 1μg/ml LPS as described in figure 3, chapter 5

Effect of cytokine environment on DX-augmented phagocytosis

Previously we have correlated augmented phagocytosis with loss of podosomes and a decrease in adhesion structures in DX-treated macrophages. We have proposed that changes in morphology and the increase in phagocytosis observed in DX-treated macrophages may in part be mediated by the decrease in p130cas expression. However the cellular morphologies of monocyte/macrophages generated by incubation in with a combination of DX and cytokines does not correspond to the cytoskeletal phenotype. We would have predicted that DX/IFNγ-treated macrophages would have a similar capacity for the engulfment of apoptotic cells as DX only treated cultures. To determine how morphological and phenotypic programming affected the phagocytic capacity of macrophages for apoptotic cells, we assessed the uptake of apoptotic neutrophils by DX and cytokine-matured macrophages. Monocyte/macrophages were cultured in 48 well plates for 5 days in the presence or absence of 1μM DX and 10ng/ml IFNγ, or 10ng/ml IL-4. Uptake of apoptotic cells by each subset of cells was assessed using plate based and flow cytometric assays as previously described. In this series of experiments the mean phagocytosis for DX-treated cultures was lower than the overall average, reflecting variability in the assay system as described in chapter 3, fig. 2. The lower levels of phagocytosis was a result of inclusion of data from two individuals which demonstrated very low levels of phagocytosis of 5.5 and 7.9% in untreated macrophages, with an increase to 16.5 and 14% respectively after DX-treatment. Hoever, treatment of monocyte/macrophages with IFNγ and IL-4 clearly reduced phagocytosis in both control and DX-treated cultures in a dose-dependent manner (fig.6). 10ng/ml IFNγ reduced basal phagocytosis in control cells from 12.2±2.3% to 4.9±1.9% respectively, and DX augmented phagocytosis from 28.6±6.3% to 8.4±2.5% (n=5-6). In a similar fashion 10ng/ml IL-4 decreased control phagocytosis from 12.2.0±2.4% to 7.2±1.0%, and DX-phagocytosis from 27.7±5.2% to 10.2±2.8% (n=5-7). Together, these findings suggest that the cytokine environment is likely to be an important factor that determines macrophage responses to corticosteroids and therefore must be carefully considered when developing strategies for DX augmentation of clearance of inflammatory cells *in vivo*.

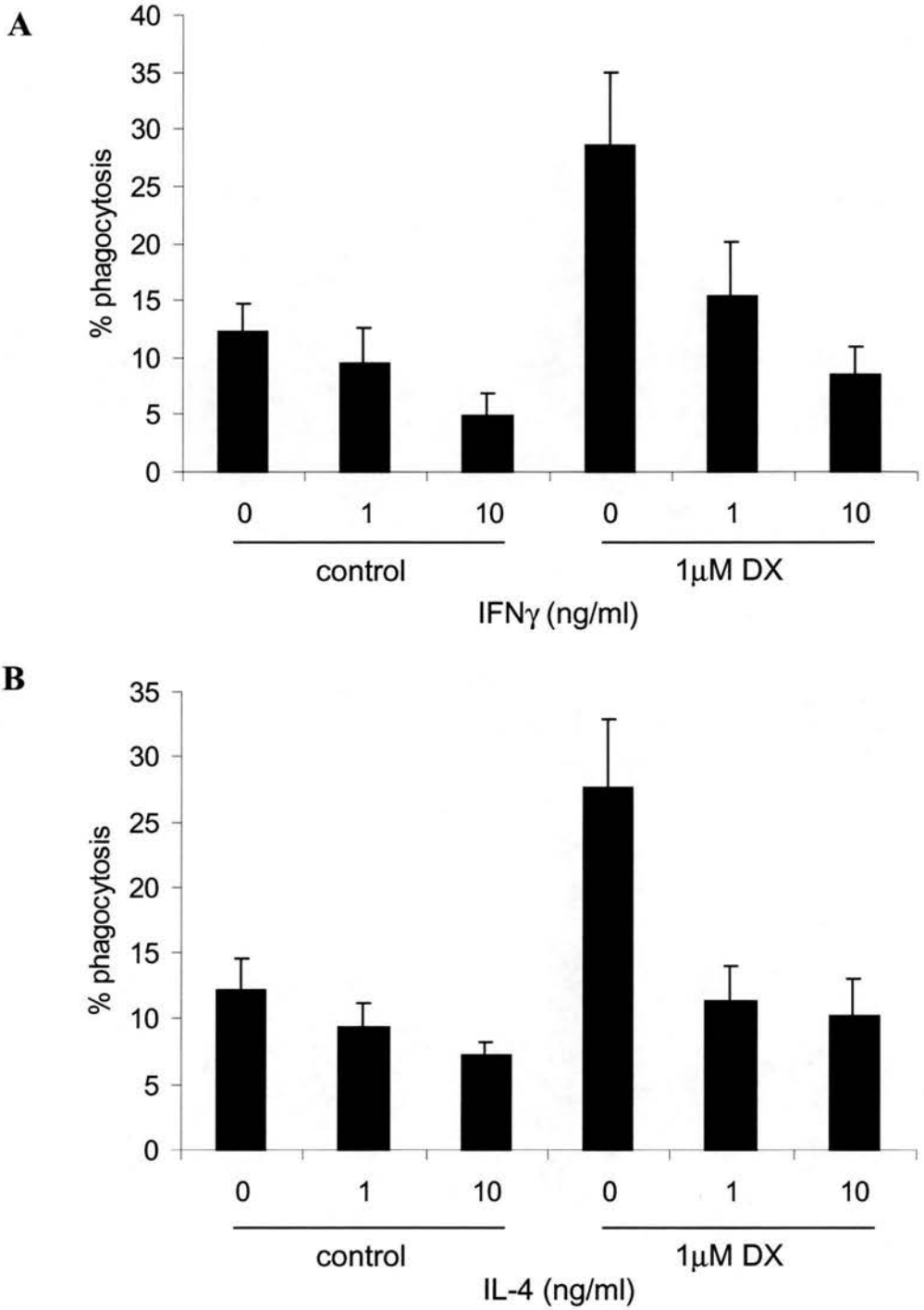

Figure 5. Effect of cytokine programming on macrophage phagocytosis of apoptotic neutrophils

Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1μM DX, 10ng/ml IFNγ or 10ng/ml IL-4 for 5 days. The capacity for phagocytosis of apoptotic neutrophils was determined using both the plate based and flow cytometric assays as described in chapter 1. Data presented as mean phagocytosis (n=5-7).

Cytokine downregulation of DX-augmented phagocytosis is not due to a decrease in "phagocytic receptors"

The presence of IL-4 and IFNγ decreased phagocytic potential in both control and DX-treated macrophages. The decrease in phagocytosis in IL-4-treated cultures correlates with the maintenance of p130cas expression, as we would have predicted if p130cas expression represented an important regulatory influence on macrophage phagocytosis. However despite the similar morphological and cytoskeletal characteristics of DX and DX/IFNγ cells, in the presence of IFNγ, DX failed to augment phagocytosis. Flow cytometric analysis revealed no change in the percentage of cells within the population expressing phagocytic receptors after treatment with cytokines. Expression of $\alpha_V\beta_3$ remained constant in all six subsets of cells. In contrast β_1-integrin expression was diminished in all conditions compared to control untreated cells. Although ligation of β_1-integrins has been reported to inhibit subsequent uptake of apoptotic cells (Erwig et al., 1999), diminished expression of β_1-integrins in IL-4 and IFNγ treated cultures did not promote phagocytosis. Despite evidence for a role for CD36 in dendritic cell phagocytosis of apoptotic cells (Albert et al., 1998), and regulation of dendritic cell maturation and function (Urban et al., 2001), binding of the pan CD36 mAb IVC7 was decreased in IL-4 and DX/IL-4 cultures. CD36 was also markedly reduced in IFNγ and DX/IFNγ treated populations to 44.9±7.8% and 15.5±5.8% mean±SEM, n=4, compared to control. However, expression of the CD36 epitope recognised by the mAb smϕ was slightly diminished in DX and DX/IFNγ, compared to control and IFNγ only, but augmented with IL-4 and to a lesser extent, DX/IL4. Expression of the glucocorticoid responsive scavenger receptor family member CD163, recognised by the mAb Ber-mac was increased in all DX-treated cultures, demonstrating dominance over cytokine influence for the expression of this molecule. Expression of CD14 was an interesting example of the balance of programming between cytokines and glucocorticoids. IL-4 reduced expression of CD14 even in the presence of DX. However, the increase in CD14 expression induced with IFNγ was inhibited by glucocorticoids. Finally, expression of the IL-4 inducible mannose receptor, was increased as expected by IL-4, but reduced below that of control by DX. Expression was further decreased in DX only cultures, and almost completely

reduced with IFNγ and DX/IFNγ. Overall, DX/IFNγ cells did not show significant differences in the decrease of receptor expression compared to DX only-treated cultures, therefore it is unlikely that the phagocytic defect observed in the presence of IFNγ can be accounted for by changes in receptor expression. Furthermore, inhibitor data for the blockade of phagocytosis in untreated and DX-treated macrophages (chapter 3, table1), suggested redundancy in receptor function, indicating that changes in receptor function would also be unlikely to account for the failure to augment phagocytosis observed after treatment with IL-4 and IFNγ in combination with DX.

DX-programmed macrophages are neither M-1 nor M-2

IFNγ and IL-4 can induce either classical or alternative macrophage "activation". In order to assess whether DX matured macrophages follow a M-1 or M-2 profile, or if glucocorticoid programming affects the cytokine-induced development of these two phenotypes (and visa versa), we next examined the expression of cell surface markers known to be differentially expressed in the presence of IFNγ and IL-4, plus the expression of arginase, a marker of alternative macrophage activation. As discussed above DX treatment did not induce surface changes consistent with either an M-1 or M-2 phenotype. Furthermore, expression of ICAM-1 and CD64 is induced with IFNγ, but expression was decreased below untreated control with IL-4 and DX. IFNγ maintains CD64 expression in the presence of DX, and glucocorticoids are able to override the effect of IFNγ on ICAM-1 expression, maintaining the downregulated phenotype.

Expression of arginase and two cysteine-rich proteins, members of the family of "resistin-like" molecules first identified in fat cells, Fizz1 and 2 in the mouse, and Fizz2 and 3 in humans have been identified as markers of "alternative" activation.(Holcomb et al., 2000; Steppan et al., 2001). To further investigate if DX-programmed cells are similar to "alternatively" activated macrophages, we assessed the level of expression of arginase and Fizz2 and 3 using RT-PCR. Monocyte/macrophages were cultured for 5 days in the presence or absence of 1μM DX and total RNA was extracted and used for RT-PCR analysis with arginase and

Fizz-specific primers. Fizz2 and 3 transcripts were detected at a similar level in untreated and DX-treated monocyte/macrophages. However arginase expression could not be detected from either untreated or DX-treated RNA (fig.7). Although the presence of non-specific bands suggests conditions were not optimal for binding, manipulation of annealing temperatures and magnesium concentrations failed to allow us to detect arginase transcripts.

		Expression (% relative to control)				
		IL-4	IFNγ	DX	DX/IL-4	DX/IFNγ
$\alpha_V\beta_3$	23C6	106.6±2.7	94.8±6.6	79.8±6.6	107±8.4	72.9±6.1
CD36	IVC7	42.3±4.1	44.9±7.8	51.0±10.8	65.0±13.3	15.2±5.8
	smϕ	153.7±44.8	120.3±29.9	81.5±26.9	131.6±57.5	77.5±22.2
CD14	UCHM1	37.0±6.9	244.1±15.1	96.9±48.3	43.1±22.5	113.3±28.4
Mannose R	19.2	145.8±0.5	7.8±1.2	38.1±1.2	85.9±2.4	21.3±0.7
β_1-integrin	P4C10	64.9±1.9	46.5±1.5	37.1±3.3	39.3±1.1	28.3±1.4
CD163	BerMac3	91.2±59.3	72.2±11.6	376.3±90.0	341.8±51.4	104.3±137.4
ICAM-1	15.2	84.2±7.5	210.1±19.1	45.4±4.2	46.7±4.6	83.0±8.1
CD64	10.1	10.2±2.5	598.3±124.6	23.0±6.6	13.3±3.0	425.2±109.2

Table 1. Effect of cytokine programming on expression macrophage cell surface molecules

Data presented as mean expression compared to control untreated macrophage populations for 3-5 experiments.

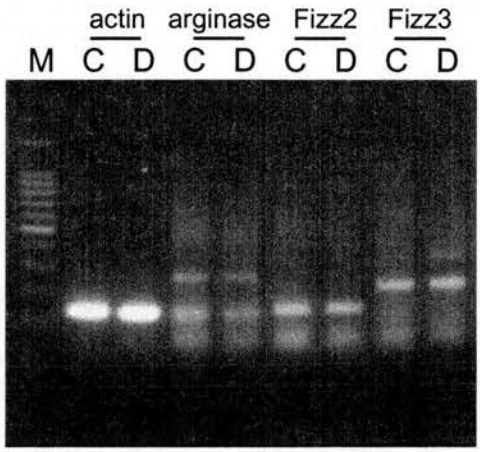

Figure 6. mRNA expression of markers of "alternative" macrophage activation
Adherent peripheral blood monocytes were cultured for 5 days in the presence or absence of 1μM DX. Total RNA from untreated and dexamethasone-treated macrophages were assessed for expression of actin, arginase, Fizz2 and Fizz3 transcripts using RT-PCR with primers described in Materials and Methods. Note the bands in arginase reactions represent non-specific products. Marker, 100 bp ladder, fragments: 1,5 kB, 1.0-0.1kb.

Discussion

DX-inhibits giant cell formation

A striking feature of DX-programming of monocyte-macrophage differentiation, was the inhibition of multinucleated giant cell formation. MNGCs are found at sites of chronic inflammation associated with granuloma formation. Granulomas can be induced by an infectious agent such as *Mycobacterium tuberculosis* (Langerhans type MNGCs), or sterile foreign bodies such as surgical implants (foreign body MNGCs), and can be initiated by cytokines such as IFNγ, IL-4 and IL-13 (for review see Anderson, 2000). In addition to the cytokine environment, a number of molecules have been proposed to be required for giant cell fusion. These include CD98 (FRP1) and α_3-integrin (FRP2) (Higuchi et al., 1998; Ohgimoto et al., 1995), CD44 (Sterling et al., 1998), the mannose receptor (DeFife et al., 1999), Macrophage Fusion Receptor (MFR) identical to P84/SHPS-1/SRPα/BIT and its ligand CD47 (Comu et al., 1997; Han et al., 2000; Jiang et al., 1999; Saginario et al., 1998; Sano et al., 1997), ICAM-1 and LFA-1 (Fais et al., 1994; Kazazi et al., 1994; Most et al., 1990), and the purinogenic receptor $P2X_7$ (Chiozzi et al., 1997). In addition, cellular filamentous actin organisation (DeFife et al., 1999; McNally et al., 1996), and an acidic extracellular environment are also thought to contribute to polykaron formation (Franklin, 1958; Hernandez et al., 1996). Little is known about the physical mechanism of monocyte fusion. Reports suggest that interaction of CD44 with an unknown ligand on the opposing cell surface, and a step wise interaction of the long splice form of MFR with CD47, followed by engagement of the short splice form of MFR, results in the close apposition of membranes. Fusion may then be induced by the putative pore-forming ability of CD47, or the pore-forming $P2X_7$ ATP transporter (Vignery, 2000). Blockade of $P2X_7$ function with pyridoxalphosphate (PPADS) has been shown to inhibit MNGC formation (Lambrecht et al., 1992).

We have assessed the involvement of a number of these molecules and aspects of their down stream signalling pathways in previous chapters. DX re-programming of monocyte differentiation appears to alter or inhibit a number of fusion mechanisms.

We were unable to demonstrate formally a requirement for $P2X_7$ for fusion of monocyte derived macrophages, as PPADS induced cell death in our system. An increase in extracellular acidity regulated by H^+-ATPases has been suggested to promote fusion (Franklin, 1958; Hernandez et al., 1996). Acidity of culture media can be approximated by inclusion of phenol red which shows a yellow colour at low pH. We observe that media pH is maintained more stringently in glucocorticoid-treated cultures compared to control cells, suggesting that ATP-regulated transporters may be altered by DX-treatment. CD47 or integrin associated protein (IAP) and CD98 have been shown to regulate integrin affinity (Brown and Frazier, 2001; Fenczik et al., 1997). CD98 ligation is thought to lead to increased LFA-1/ICAM-1 adhesion in lymphocytes via the activation of Rap1 (Suga et al., 2001). In monocytes it is thought to induce a pathway involving ERK activation (Miyamoto et al., 2000). Preliminary evidence suggests CD98 expression is slightly elevated with DX-treatment, although downregulation of ERK activity in DX-treated cells could potentially compromise CD98 signalling. Occupancy of CD44 by ligands such as hyaluronan, chondroitin sulfate, osteopontin or recombinant CD44 prevents fusion, due to an inhibition of CD44 mediated cell-cell interaction (Sterling et al., 1998). Glucocorticoid treatment downregulates CD44 expression (chapter 3, table 1), that may mimic the sequestration of CD44 by ligand occupancy, demonstrated to inhibit giant cell formation. MFR was cloned as a fusion regulating protein by Vignery and colleagues, and was found to be identical to the scaffolding protein SHPS-1, involved in adhesion signalling (Timms et al., 1999). Pathways that promote cellular awareness of the substratum may therefore also be employed to recognise cell-cell contact. Two of the major SHPS-1 interacting proteins thought to mediate downstream signalling are Pyk2, and SHP-1, which show reduced phosphorylation and expression in DX-treated macrophages (chapter 4, fig. 5). Dysregulation of SHPS-1 signalling may also contribute to the lack of polykaryon formation in DX-treated monocyte/macrophage cultures.

Polarization and the formation of highly adherent monocyte aggregates is thought to promote fusion via the mannose receptor (DeFife et al., 1999), however DX-treated macrophages do not appear polarised, have a disorganised actin cytoskeleton and

show decreased mannose receptor expression. Similarly localisation of ICAM-1 on the monocyte cell surface to uropod-like structures is thought to promote fusion by interaction with LFA-1 (Fais et al., 1997; Fais et al., 1994; Kazazi et al., 1994; Most et al., 1990). Although the expression of LFA-1 in response to DX-programming has not been assessed, glucocorticoids down regulate the expression of ICAM-1 on 5-day macrophages. The time point during maturation at which this occurs has not been established, however if it were early during maturation whilst monocyte/macrophages were at their most fusogenic (Most et al., 1997), it would provide a further mechanism for glucocorticoid inhibition of MNGC formation.

The pro-inflammatory cytokine IFNγ has been shown to induce MNGC formation *in vitro* and *in vivo*. Crohn's disaese and sarcoidosis, chronic inflammatory conditions associated with granuloma formation, show increased production of IFNγ at the inflammatory lesion and in the periphery (Fais et al., 1994; Robinson et al., 1985). Peripheral IFNγ is likely to be generated by T_H1 type T-cells. Exposure of monocyte/macrophages to T_H1 cytokines would induce an M-1 response with the further release of pro-inflammatory cytokines including IFNγ potentiating granuloma formation. Thus the refractivity of DX-treated macrophages to IFNγ induced fusion, and possibly aspects of the M-1 phenotype could explain the effectiveness of glucocorticoid therapy for granulomatous conditions such as sarcoidosis (Gibson, 2001). The mechanism of initiation of granuloma formation in conditions such as sarcoidosis and Crohn's is not known but appears to occur in the absence of any known antigen (Sheffield, 1990). MNGC have the capacity to present antigen as they have been reported to express MHCII and B7 (Burgio et al., 1995), however polykaryons are poorly phagocytic (Mariano and Spector, 1974, plus our own *in vitro* observations). It has been proposed that during membrane fusion, exogenous antigens and membrane molecules such as ICAM-1 may be included into the MNGC cytoplasm and presented (Fais et al., 1997). MNGCs therefore have a capacity to present endogenous antigens promoting autoimmune responses.

Infection of monocytes and T cells by the HIV virus induces a similar fusion mechanism as proposed for IFNγ, utilising an ICAM-1 mediated pathway, with the

formation of MNGCs and syncitia respectively (Butini et al., 1994; Fais et al., 1996). MNGCs are able to survive in culture for more than 2 years (Bigi et al., 1990), fusion of infected monocytes would therefore provide a long-lived reservoir of viral production. In contrast, syncitia formation occurs by the rapid recruitment and fusion of hundreds of cells which die shortly after infection (Lifson et al., 1986; Sodroski et al., 1986). Formation and turnover of syncitia may explain why they are rarely detected in the organs of AIDS patients. More importantly the rapid demise of syncitia proposes a mechanism for the rapid depletion of $CD4^+$ cells in AIDS patients (Soll and Kennedy, 1994). Conventional glucocorticoid therapy would be detrimental to T cell survival, promoting further immune suppression. Understanding the mechanism of glucocorticoid inhibition of cell fusion may provide novel approaches for inhibition of long-lived cellular reservoirs for viral production, and the depletion of T-cells by syncitia formation.

Osteoclasts, cells of the monocyte/macrophage lineage are also formed by the cell fusion of granulocyte-macrophage progenitors. Osteoclasts are found at bone surfaces where they mediate bone remodelling. Over-activity of osteoclasts and increased bone resorption has been implicated in osteoporosis, a complication observed during long term glucocorticoid therapy. However based upon our *in vitro* observations we would predict that DX would inhibit cell fusion and hence decrease osteoclast formation leading to osteopetrosis characterised by increased bone deposition. Additionally the disruption of podosomes may decrease the rate of bone turnover and remodelling following DX-treatment. We have only assessed the affect of DX on giant cell formation in response to IFNγ, and IL-4, and therefore cannot predict if DX would inhibit fusion in response to other soluble mediators. The role of IFNγ and IL-4 in osteoclast formation is unclear with contradictory findings *in vitro* (Anderson, 2000; Vignery et al., 1990). However ICAM-1 and LFA-1 have been implicated in the fusion process (Kurachi et al., 1993) and TNFα as the soluble mediator inducing osteoclast formation and bone resorption activity (Merkel et al., 1999; Takahashi et al., 1999). Our own experiments have demonstrated that DX-programmed macrophages can respond to TNFα in terms of an induction of a MAPK response, therefore macrophage TNFα responses may not be compromised by

glucocorticoid treatment. Interestingly osteoblast viability can be modulated by glucocorticoids. Corticosterone has been shown to induce mouse osteoblast cell death (Gohel et al., 1999). Loss of osteoblasts would lead to the development of an osteopetrotic phenotype. In contrast, DX has been shown to inhibit TNFα mediated osteoblast apoptosis (Chae et al., 2000). Glucocorticoids therefore appear to influence a number of important processes involved in bone homeostasis.

DX-inhibits IL-4 programming of dendritic-like cells

Culture of peripheral blood monocytes in GM-CSF and IL-4 induces the formation of dendritic cells (Romani et al., 1994; Sallusto and Lanzavecchia, 1994). Glucocorticoids have been shown to inhibit aspects of dendritic cell maturation and function. DX-treatment of dendritic cell progenitors during differentiation induces a subset of $CD14^+$ $CD83^-$, and $CD14^+$ $CD80^-$ cells distinct from both immature and mature dendritic cells (Canning et al., 2000; Matasic et al., 1999). Additionally glucocorticoids have been proposed to promote the formation of macrophages rather than dendritic cells from common precursosrs (Matasic et al., 1999; van den Heuvel et al., 1999). Glucocorticoids have been reported to decrease the uptake and processing of antigen, but not presentation by some groups (Holt and Thomas, 1997). However, conflicting reports demonstrate an increase in mannose receptor-mediated endocytosis (Piemonti et al., 1999a), and a down regulation of antigen presentation and expression of costimulatory molecules CD80 and CD86 (Moser et al., 1995; Sacedon et al., 1999; Vanderheyde et al., 1999). Differences in the origin and the maturation status of dendritic cells exposed to steroid may account for these discrepancies. DX treatment of monocytes retarded their differentiation into an immature dendritic cell phenotype, whereas DX could not block the subsequent differentiation from immature to a mature dendritic cell (Piemonti et al., 1999b). Furthermore, in contrast to the effects of DX on mature dendritic cells treatment of immature dendritic cells with DX prior to the maturation stimulus LPS, resulted in a decrease in priming of T lymphocytes for the production of IFNγ, and multiple re-stimulation of lymphocytes with DX/LPS treated dendritic cells resulted in the production of T regulatory cells (Matyszak et al., 2000).

Cytokines inhibit DX-augmented phagocytosis

It might be expected that cytokines associated with resolving inflammation would potentiate clearance of apoptotic cells. IL-4, IL-10 and IL-13 which antagonise the effects of IFNγ and other type 1 cytokines are associated with the healing phase and have the capacity to "alternatively" activate macrophages (Goerdt and Orfanos, 1999). Interestingly, glucocorticoids have also been described to induce "alternative" activation. However, uptake of apoptotic cells was decreased with both cytokines. Previous conflicting reports relating to the effects of IL-4 and other T_H2 cytokines (Erwig et al., 1998; Ren and Savill, 1995) may reflect the species and tissue origin of macrophages used in the studies and the period of cytokine exposure. Long term exposure to cytokines used in this study will alter monocyte differentiation pathways making direct comparisons with previous studies difficult. For example our data indicates that long term and short term steroid exposure augments phagocytosis in different ways (Giles et al., 2001). Interestingly IL-4 has recently been shown to induce the expression of 11β-hydroxysteroid dehydrogenase type1 (11β-HSD1) which metabolises the inactive glucocorticoid cortisone to the active cortisol in monocyte/macrophages (Thieringer et al., 2001). This could potentially promote clearance of apototic cells *in vivo*. However, our results suggest that the presence of IL-4 would abrogate any effects of glucocorticoids on macrophage function. This may be due to the induction of a dendritic cell like phenotype by IL-4 as described in the previous section. Differences in cross-presenting capacity between macrophages and immature dendritic cells may be due to the engagement of different intracellular pathways upon internalisation, which may influence the efficiency of particle uptake. Furthermore, the intracellular pathway engaged might determine the cellular response, such as release of soluble mediators, which could influence particle processing by an autocrine mechanism. For example, IL-10 and IL-13 released by macrophages after apoptotic cell ingestion upregulate class II expression promoting presentation via this pathway. The cytokine profile generated by dendritic cells after phagocytosis of apoptotic particles has not been assessed, but may contrast profiles observed in the macrophage. Additionally the direct effect of glucocorticoids on dendritic cell phagocytosis of apoptotic cells has not been assessed, glucocorticoid inhibition of dendritic cell maturation may

prevent expression of co-stimulatory molecules during augmented apoptotic cell uptake. We are currently assessing the effect of DX and DX/IL-4 programming on the expression of co-stimulatory molecules CD80 and CD86. Efficient uptake and presentation of pathogens and opsonized particles is required for a functional immune response. It would therefore be interesting to assess the capacity of IL-4 or DX/IL-4 treated macrophages to phagocytose opsonized erythrocytes or complement opsonized zymosan.

Loss of p130cas in DX-treated cells was suggested to contribute to podosome loss and increased phagocytic capacity (chapter 4). Expression of p130cas was apparent in DX/IL-4 treated cells, although the adhesions appeared slightly different to classical posodomes, it correlated with decreased phagocytosis. In contrast DX/IFNγ treated cells had no podosome structures or p130cas expression, but also had decreased phagocytic capacity. Comparison of DX/IFNγ and DX matured macrophages provide a strategy for the elimination of genes involved in morphological changes alone, allowing rapid identification of modulators of macrophage phagocytosis. During manipulation of cells, for example washing during phagocytosis assays, or during detachment for phenotyping experiments, it was evident that DX/IFNγ macrophages were much less adherent than cells treated only with DX. Although we hypothesise that the reduction of adhesion processes observed in DX-treated macrophages potentiates their phagocytic capacity, too little adhesion, as seen with cells treated with cAMP may inhibit phagocytosis. Interestingly, IFNγ treated macrophages that expressed no p130cas but had extensive podosome formation and decreased phagocytosis, may provide a useful model system for the study of other molecules involved in adhesion and phagocytosis. For example assessment of Rac activity in DX/IFNγ and DX/IL-4 treated cells would be most interesting. Additionally both glucocorticoid and cytokine matured macrophages expressed WASp, a protein shown to be required for phagocytosis and podosome formation (Leverrier et al., 2001; Linder et al., 2000; Linder et al., 1999). Thus IFNγ matured cells provide a model system in which function of WASp may be assessed, such that its expression is sufficient for podosome formation, but cannot rescue phagocytosis.

M-2 or not M-2 ? that is the question

Phenotypically DX-treated monocyte/macrophages are distinct from either IFNγ or IL-4 treated cells. IFNγ upregulates the expression of CD14, ICAM-1 and CD64, and downregulates mannose receptor expression. Conversely IL-4 treatment induces mannose receptor expression, and down regulates CD14, ICAM-1 and CD64. The profile of these molecules after DX-treatment shows similarities to both M-1 and M-2 cells, with the decrease in ICAM-1 and CD64 seen after IL-4 treatment, but a decrease in mannose receptor expression characterised by IFNγ. The effect of glucocorticoid programming is dominant over the cytokine influence in terms of ICAM-1 and mannose receptor expression, but it is unable to prevent IFNγ induced CD64 expression, or IL-4 induced CD14 downregulation. Based upon these data, I propose that DX-matured macrophages are clearly not "alternatively" activated. Furthermore, in glucocorticoid-treated cultures we were unable to detect augmented expression of mRNA encoding arginase or changes in transcripts encoding the recently identified markers of alternative activation: Fizz2 and Fizz3. Examination of other specific functions of DX-treated cells may allow better definition of this phenotype. The effect of early exposure to DX on monocyte/macrophage differentiation supports the suggestion that initial cytokine exposure determines macrophage phagocytic function (Erwig et al., 1998). Therefore, during an inflammatory reaction extravasating monocytes will have already been exposed to elavated levels of glucocorticoids present in the bloodstream in response to stress stimuli prior to encountering the cytokine environment at the inflammatory site. Monocyte/macrophages may therefore be unable to be programmed as M-1 or M-2, producing a third subset of macrophages typified by our DX-treated monocyte/macrophage phenotype generated during *in vitro* culture. Such effects may have profound implications for patients receiving long-term glucocorticoid therapy.

CHAPTER 8: SUMMARY AND FUTURE DIRECTIONS

The aim of this study was to understand the mechanisms underlying the regulation of macrophage phagocytosis of apoptotic cells, a possible "rate limiting step" for resolution of inflammation. Much research work has concentrated on modulation of granulocyte death programs, however in the absence of clearance, there is a persistence of inflammatory infiltrate and chronic inflammation occurs. The recent reports describing how the act of engulfment triggers the final steps of the apoptotic program in the nematode worm, demonstrate the evolutionary conservation of clearance mechanisms in the maintenance of tissue homeostasis.

When this work was originally undertaken, little was known of the intracellular pathways regulating phagocytosis of apoptotic cells and research was centred largely on understanding the interaction of the phagocyte and prey at the receptor level. However, the diversity of receptor families involved suggested redundancy in the system and therefore regulation at this level would not present a target for therapeutic intervention. We therefore first characterised the effects of a modulator of macrophage phagocytic capacity for apoptotic cells, glucocorticoids. Although previous work had demonstrated an upregulation in phagocytosis of apoptotic cells after 24 hours of glucocorticoid treatment, preliminary experiments suggested that this increase could be further augmented with longer exposure. We demonstrated that exposure of monocytes to glucocorticoids immediately following isolation for the full period of their maturation into macrophages not only increased phagocytosis but also brought about a number of changes in the resultant monocyte/macrophage. In summary, long term dexamethasone treatment:

- Increased phagocytosis of apoptotic cells four-fold
  - Phagocytic index was increased nearly seven fold
  - Augmentation was independent of increases in receptor expression or function
- Uptake of opsonized particles was also increased
- DX-treated macrophages showed a distinct morphology

- Increased homogeneity, smaller, rounder
- Decreased formation of adhesion processes
- Disordered actin cytoskeleton

- Biochemical analysis suggested this was linked to changes in adhesion signalling
  - Decrease in Paxillin and Pyk2 phosphorylation
  - Decrease in p130cas expression
  - Increase in Rac activity
  - Decreased basal ERK activity
- The distinct morphological, biochemical and functional characteristics of DX-treated macrophages were influenced by the cytokine environment
  - IFNγ and IL-4 inhibited DX-augmented phagocytosis without influencing morphology

Our hypothesis generated from this data was that the increased cytoskeletal plasticity, from decreases in adhesive processes involved in cell substratum interactions, and augmented Rac activity allowed the efficient rearrangement of cytoskeletal components required for the uptake of apoptotic cells. We proposed that decrease in p130cas was pivotal in generating this phenotype due to its role in podosome formation and interaction with DOCK180 and Crk. The number of potential binding partners for Crk and DOCK180, suggest that some of these cytoskeletal regulators may preferentially be involved in adhesion, whilst others may be required for phagocytosis. We propose that the balance of expression of these molecules is pivotal to the overall functional outcome, in a manner analogous to the balance of pro- and anti-apoptotic Bcl-2 family members determining the sensitivity of cells to apoptotic stimuli. Thus the balance of adaptor proteins may determine a cells phagocytic potential. For example the absence of p130cas in glucocorticoid treated macrophages may therefore promote interaction of Crk and DOCK180 with the ELMO family of adaptor proteins recently implicated in phagocytic uptake in *C. elegans* (Gumienny et al., 2001).

With the publication of work by Albert et al (2000) and Tosello-Trampont (2001) there has been a shift in emphasis of research in this field to understanding the role of

molecules involved in regulating the actin cytoskeleton in the uptake of apoptotic cells, rather than events on the cell surface. The data presented in this thesis supports the suggestion that cytoskeletal regulation in the clearance of apoptotic cells, is important. However, my data suggests also that there are additional complexities. The cytokine and cytokine/DX maturation experiments generated several interesting "subsets" of macrophage cytoskeletal phenotypes described in chapter 7. In summary:

- IFNγ; podosomes, no p130cas
- DX/IFNγ; no podosomes, no p130cas
- IL4; podosomes, p130cas
- DX/IL-4; no podosomes, p130cas

The common factor between these four treatments is that unlike DX alone they all displayed low phagocytic potential for apoptotic cells. These experiments raise questions as to the validity of our suggested target molecule p130cas indicating it is unlikely to be the only key regulator of phagocytic capacity. However, we would be naïve to expect such a complex process to be influenced by a single molecule. Further development of the TAT fusion protein technology described in chapter 6 will allow us to determine the contribution of p130cas expression to the DX phenotype. The generation of the cell subtypes following combinations of DX and cytokine provides a valuable tool for divorcing adhesion from phagocytosis. Array technology will allow gene expression profiling that may provide important clues as to the key regulatory elements involved in phagocytic clearance of apoptotic cells. However, we must be aware that focussing on single changes might be misleading.

What may be other targets for glucocorticoid modulation? As mentioned above the recent identification of ELMO, the human homologue of the *C. elegans* protein CED-12, and its role in phagocytosis provides a potential target for modulation during DX-re-programming. Decrease in p130cas and an increase in ELMO may promote phagocytosis as opposed to adhesion. Within the context of molecules evaluated in this study, Pyk2 is worth further investigation. Pyk2 has been shown to interact with Crk and can influence ERK activity, one "marker" of DX re-programming, and has been linked to regulation of podosome formation.

Interestingly, although both IFNγ and DX-treated macrophages lack p130cas and express WASp, the ability to form podosome adhesions is lost in the presence of glucocorticoid, suggesting that organisation in these phenotypes is dependent on signalling upstream of these molecules. Assessment of Pyk2 phosphorylation status in cytokine-treated macrophages may therefore prove very informative. It was demonstrated several years ago that tyrosine kinase inhibitors block the uptake of apoptotic cells. The identification of a new phagocytic receptor, Mer, as a receptor tyrosine kinase, a putative pTyr motif in the newly cloned human PS receptor, and the association of syk with CrkL and WASp, suggests that membrane associated or membrane localised tyrosine kinases may have an important part to play in the regulation of engulfment. We tried (unsuccessfully) to immunoprecipitate Crk and assess its phosphorylation status in DX treated cells. It remains possible that tyrosine phosphorylation is required for the control of interaction of Crk with a number of adaptor molecules. My studies also illustrate an important conceptual point. Documenting altered gene expression patterns in macrophages deficient in phagocytic function, although informative, cannot identify subtle post-translational modifications for example phosphorylation or glycosylation, that are critical for function. Biochemical approaches will therefore be required to fully understand the mechanisms that regulate phagocytic potential.

The studies in this thesis have been carried out using isolated cells entirely *in vitro*. Although this approach allows the intricate dissection of signalling pathways, it cannot reconstitute the specialised microenvironments that may further influence phagocytic potential *in vivo*. For example adhesion to different matrices or ligation of macrophage cell surface molecules can have a profound effect on macrophage phagocytic function (McCutcheon et al., 1998, Hart et al., 1997). Furthermore, work presented in chapter 7 illustrated the potential effect of the cytokine environment on phagocytosis. Clearly, glucocorticoid therapy in isolation may not be the most effective approach for augmenting phagocytosis in many inflammatory conditions. Combination of glucocorticoid and anti-cytokine treatment may provide a strategy for more efficient clearance of inflammatory infiltrate. This suggestion would require testing in models of inflammation using different inflammatory stimuli in

different tissues. However, such combinations may allow a beneficial reduction in steroid doses, decreasing the detrimental effects of long-term glucocorticoid therapy.

Finally, one question concerning the effect of glucocorticoid programming on p130cas expression not addressed by this study is whether the effects are cell specific or does downregulation of p130cas expression occur in other cell types? Sequence analysis demonstrated that p130cas was identical to BCAR1 (Breast Cancer Anti-oestrogen Resistance), identified in tamoxifen-resistant breast carcinoma cells (Brinkman et al., 2000). Increased expression levels of BCAR1/p130cas in breast cancer cell cytosols correlated with rapid recurrence of disease (van der Flier et al., 2001). A high BCAR1/p130Cas level was also associated with a higher likelihood of resistance to first-line tamoxifen treatment in patients with advanced breast cancer (van der Flier et al., 2000). Given the close link between adhesion and resistance to apoptotic stimuli and the potential for secondary metastases,
it may be that p130cas expression has a pivotal role in controlling tumour cell growth. Glucocorticoid induced changes in ERK activity that are required for proliferation and migration in certain cell types could also contribute to the modulation of tumour growth. Potential targeting of glucocorticoids to cancerous tissue in combination with anti-oestrogen treatment may provide a novel therapy of breast carcinoma.

In conclusion, this study has provided a novel insight into the control of monocyte-macrophage differentiation, adhesion and phagocytosis. The data presented in this thesis suggests a major impact of long term glucocorticoid treatment on cell type that is pivotal to immune regulation and processes involved in the resolution of inflammation.

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APPENDIX A

p130cas nucleotide sequence

```
   1 gaggcggcag ctgcgcggcg gcaccggggc ggctgcggcg cgctcggagc cccgaggcac
  61 gcggcccggg cagctcggtg tgcgcccccg cgagagccgg gccccaggcc cgccggacac
                START →
 121 catgaaccac ctgaacgtgc tggccaaagc gctctatgac aatgtggccg agtccccgga
 181 tgagctctcc ttccgcaagg gtgacatcat gacggtgctg gagcaggaca cgcagggcct
 241 ggacggctgg tggctctgct cgctgcatgg gcgccagggc atcgtgcctg ggaaccgcct
 301 caagatcttg gtgggcatgt atgataagaa gccagcaggg cctggctccg gccctcccgc
                      g
 361 caccccggcc cagcctcagc ctggcctcca tgccccagcg cctccggcct cccagtacac
                                                      g
 421 gcccatgctc cccaacacct accagcccca gccagacagc gtctacctgg tgcccactcc
 481 cagcaaggct cagcaaggcc tctaccaagt cccgggtccc agccctcagt tccagtctcc
 541 cccagccaag cagacatcca ccttctcgaa gcagacaccc catcacccgt ttcccagccc
 601 ggccacagac ctgtaccagg tgcccccagg gcctggaggc cctgcccagg atatttacca
 661 ggtgccacct tctgccggga tggggcatga catctaccag gtccccccgt ccatggacac
 721 acgcagctgg gagggcacga agcccccggc aaaggtggtg gtgcccaccc gcgtggggca
                                          Primer 2 →
 781 gggctatgta tacgaggccg cccagccgga gcaggacgag tacgacatcc cgcgacacct
 841 gctggccccg gggccacagg acatctatga tgtgcccccg gttcgggggc tgcttcccag
 901 ccagtatggc caggaggtgt atgacacacc ccccatggct gtcaagggtc ccaatggccg
 961 agacccgttg ctggaggtgt atgacgtgcc ccccagtgtg gagaagggcc tgccaccgtc
1021 caaccaccac gcagtctacg acgttcctcc atcggtgagc aaggatgtgc ccgatggccc
                ← Primer 3
1081 actgctgcgt gaggagacct acgatgtgcc ccccgccttc gccaaggcca agcccttga
1141 cccggcccgc accccactgg tactggctgc gccccctcca gactccccgc cggccgagga
1201 cgtgtatgac gtgccgcccc cggctcctga cctctacgac gtgccccctg gcttgcggcg
                                          Primer 4 →
1261 gcctggcccg ggcaccctgt acgatgtgcc ccgtgaacgg gtgcttcctc ctgaggtggc
1321 tgatggtggc gtggtcgaca gtggtgtgta tgcggtgcct cccccagctg aacgtgaagc
1381 cccggcagag ggcaagcgcc tgtcggcctc cagcaccggc agcacacgca gcagccagtc
1441 tgcgtcctcc ttggaggtgg cagggccggg ccgggaaccc ctggagctgg aagttgctgt
                t
1501 ggaggccctg gcacggctgc agcagggtgt gagcgccacc gttgcccacc ttctggacct
1561 ggcaggcagc gccggtgcga ctgggagctg gcgtagcccc tctgagccac aggagccgct
1621 ggtgcaggac ctgcaggctg ctgtggccgc tgtccagagt gccgtccacg agctgttgga
1681 gtttgcccgc agcgcggtgg gcaatgctgc ccacacatct gaccgtgccc tgcatgccaa
                                          ← Primer 5
1741 gcttagccgg cagctgcaga agatggagga cgtgcaccag acgctggtgg cacatggtca
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1981 cctgcacccc aaccccactg acaagaccag cagcatccag tcacgacccc tgccctcacc
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2221 gctgaagcag tttgaacgac tggaacagga ggtgtcacgg cccatagacc acgacctggc
2281 caactggacg ccagcccaac ccctggcccc ggggcgaaca ggcggcctgg ggccctcgga
                a                         ← Primers 6 and 7 →
2341 ccggcagctg ctgctcttct acctggagca gtgtgaggcc aacctgacca cactgaccaa
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2401 cgccgtggac gccttcttta ccgccgtggc caccaaccag ccgcccaaga tctttgtggc
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3181 tccatttaaa taaagttttt aaaaggaa
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STOP

p130cas amino acid sequence

SH3

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START          MNHLNVLAKALYDNVAESPDELSFRKGDIMTVLEQDTQGLDGWW
                                                        R
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                A
LPNTYQPQPDSVYLVPTPSKAQQGLYQVPGPSPQFQSPPAKQTSTFSKQTPHHPFPSP

ATDLYQVPPGPGGPAQDIYQVPPSAGMGHDIYQVPPSMDTRSWEGTKPPAKVVVPTRV
                                Substrate Domain
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KAKPFDPARTPLVLAAPPPDSPPAEDVYDVPPPAPDLYDVPPGLRRPGPGTLYDVPPRE

RVLPPEVADGGVVDSGVYAVPPPAEREAPAEGKRLSASSTGSTRSSQSASSLEVAGPG
                L
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AAVQSAVHELLEFARSAVGNAAHTSDRALHAKLSRQLQKMEDVHQTLVAHGQALDAGR

GGSGATLEDLDRLVACSRAVPEDAKQLASFLHGNASLLFRRTKATAPGPEGGGTLHPN
          Proline rich motif                src phosphorylation motif
PTDKTSSIQSRPLPSPPKFTSQDSPDGQYENSEGGWMEDYDYVHLQGKEEFEKTQKEL

LEKGSITRQGKSQLELQQLKQFERLEQEVSRPIDHDLANWTPAQPLAPGRTGGLGPSD
      Q
RQLLLFYLEQCEANLTTLTNAVDAFFTAVATNQPPKIFVAHSKFVILSAHKLVFIGDT

LSRQAKAADVRSQVTHYSNLLCDLLRGIVATTKAAALQYPSPSAAQDMVERVKELGHS

TQQFRRVLGQLAAA
```

Sequence shown as original clone with nucleotide and subsequent amino acid changes shown above the sequence. Putative phosphorylation motifs in the amino acid sequence are shown in grey font.

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Review

AN APPETITE FOR APOPTOTIC CELLS? CONTROVERSIES AND CHALLENGES

The biochemical pathways involved in programmed cell death are broadly similar in phylogenetically diverse multicellular organisms, including the nematode worm *Caenorhabditis elegans*, *Drosophila* and mammals, suggesting that cell death and subsequent phagocytosis of apoptotic cells represents an important regulatory mechanism that has been conserved through evolution (Ellis *et al*, 1991; Hengartner, 1996).

During apoptosis, the cell activates intrinsic suicide mechanisms that lead rapidly (within hours) to the characteristic macroscopic features of cell shrinkage, chromatin condensation, membrane budding and, eventually, the formation of one or more apoptotic bodies (Kerr *et al*, 1972). Phagocytosis of senescent cells had been described in the late nineteenth century by the Russian biologist Elie Metchnikoff who observed that 'microphages' (neutrophil granulocytes) were 'englobed' by macrophages in injured tadpole fins, although the significance of these observations has only become apparent in the last few decades. It has now been established that apoptotic cells are swiftly recognized and ingested by neighbouring phagocytes without elicitation of pro-inflammatory responses from phagocytic cells (Meagher *et al*, 1992; Savill *et al*, 1993; Fadok *et al*, 1998). Apoptosis and the subsequent phagocytic clearance of senescent cells have central roles in many fundamental biological processes, including normal tissue turnover (Han *et al*, 1993), remodelling of embryological tissues (Hopkinson-Woolley *et al*, 1994), development of the immune system (Cohen, 1991) and the normal resolution processes of inflammation (Haslett *et al*, 1994). Macrophages are the principal phagocytes that are responsible for clearance of apoptotic cells in mammals, although cells with the potential for phagocytosis, e.g. endothelial cells (Dini *et al*, 1995; Hess *et al*, 1997), fibroblasts (Hall *et al*, 1994), glomerular mesangial cells (Hughes *et al*, 1997), hepatocytes (Dini *et al*, 1992), Sertoli cells (Shiratsuchi *et al*, 1997) and vascular smooth muscle cells (Bennett *et al*, 1995) may remove apoptotic cells in certain circumstances.

GRANULOCYTE APOPTOSIS AND THE RESOLUTION OF INFLAMMATION

There is increasing evidence that aberrant apoptosis and/or phagocytic clearance of apoptotic granulocytes may contribute to the pathogenesis of inflammatory diseases (Fig 1). Neutrophil granulocytes play a vital role in the body's defence against infectious agents, but uncontrolled release of their formidable array of toxic substances may inflict 'friendly fire' damage on surrounding tissue and propagate the inflammatory response (Haslett *et al*, 1994). In contrast to the potentially harmful effects of necrotic cell death upon tissue integrity and organ function, deletion of cells by apoptosis represents a cellular removal pathway that does not provoke pro-inflammatory phagocyte responses. Indeed, dysregulated neutrophil and eosinophil granulocyte apoptosis has been implicated in the pathogenesis of the adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis, ulcerative colitis, rheumatoid arthritis, asthma and other allergic diseases (Weiss, 1989; Meagher *et al*, 1992; Haslett *et al*, 1994; Stern *et al*, 1996; Woolley *et al*, 1996). Uncoupling of neutrophil effector responses associated with apoptosis (Whyte *et al*, 1993), together with 'anti-inflammatory' clearance by phagocytes, provides a mechanism for the 'safe' disposal of potentially destructive inflammatory cells. Strategies for manipulation of cellular apoptosis programmes for therapeutic gain in inflammatory or allergic disease are likely to fail if the capacity for clearance of apoptotic cells is exceeded. Data from *in vivo* experimental models support this hypothesis. For example, induction of widespread apoptosis in the liver, after treatment of mice with intraperitoneal Fas antibody, led to extensive hepatic necrosis and death of the animals (Ogasawara *et al*, 1993). Similarly, induction of bronchial and alveolar epithelial cell apoptosis in the rat lung by repeated inhalation of aerosolized Fas antibody culminated in pulmonary inflammation and scarring (Hagimoto *et al*, 1997). Although previous strategies have identified several distinct families of molecules involved in apoptotic cell recognition, the precise mechanism of phagocytosis of apoptotic cells is still not known. Clearly, a detailed understanding of the mechanisms of phagocytic clearance of apoptotic cells and the underlying regulatory mechanisms are likely to have important implications for the design of therapeutic strategies for the treatment of inflammatory disease. In this review, we will illustrate some of the problems that need to be resolved and describe some of the challenges for future research on phagocyte recognition of apoptotic cells.

PHAGOCYTE RECEPTORS FOR RECOGNITION OF APOPTOTIC CELLS

At least seven distinct molecular families have been implicated in the recognition process. Table I summarizes the experimental data that support the involvement of the receptor pathways for the clearance of apoptotic cells shown schematically in Fig 2 (see also Savill *et al*, 1993; Hart *et al*,

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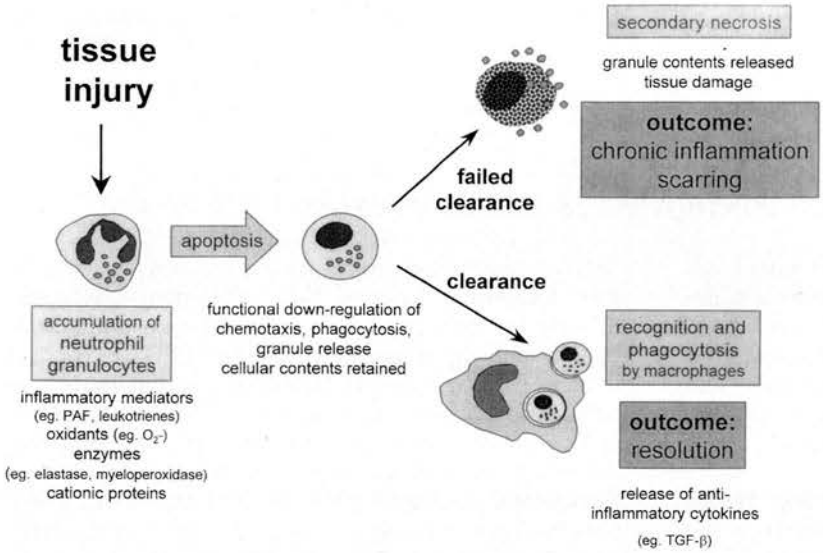

Fig 1. Schematic representation illustrating the proposed role of neutrophil apoptosis and phagocytic clearance by macrophages in the process of resolution of inflammation.

1996; Platt *et al*, 1998a). The first molecular pathway for the specific clearance of apoptotic neutrophils by human macrophages was identified on the basis of distinct patterns of inhibition by carbohydrates and sensitivity to altered pH (Savill *et al*, 1989). Subsequent analyses implicated the phagocyte integrin, $\alpha v\beta 3$ (Savill *et al*, 1990) and the class B scavenger receptor, CD36, as receptors of apoptotic cell-associated thrombospondin (Savill *et al*, 1992). Inhibition of phagocytosis of apoptotic cells after blockade of integrin function in murine bone marrow-derived macrophages indicated that similar pathways exist in other species (Fadok *et al*, 1992a). However, Fadok *et al* (1992b) also demonstrated that murine peritoneal macrophages did not use this pathway and characterized phosphatidylserine (PS) as a ligand for a putative PS receptor. In support of the suggestion that macrophages from different tissue sites use distinct pathways, Kupffer cells recognize altered carbohydrate present on apoptotic cells via the asialoglycoprotein receptor (Dini *et al*, 1995), whereas mouse thymic macrophages use class A scavenger receptors (Platt *et al*, 1998b). Furthermore, alteration of macrophage phenotype after stimulation (for example, with digestible particles) may drive switching of the dominant pathway used for clearance (Fadok *et al*, 1993; Pradhan *et al*, 1997). A number of alternative pathways for human macrophage clearance of apoptotic cells have been reported. Detailed characterization of macrophage recognition of apoptotic B cells (Flora & Gregory, 1994) pointed to a role for phagocyte CD14 (Devitt *et al*, 1998) and apoptotic cell CD50 (Moffatt *et al*, 1999). Recent data have also suggested a role for elements of humoral immunity, specifically, complement components C1q (Botto *et al*, 1998) and C3bi (Mevorach *et al*, 1998). Specific opsonization of apoptotic cells may serve to accelerate apoptotic cell clearance. In addition, there are other receptors that have been identified to have a role in apoptotic cell clearance, e.g. ATP binding cassette transporter (Luciani & Chimini, 1996). However, whether these molecules mediate phagocyte recognition of apoptotic cells directly is unclear. These data serve to illustrate the molecular complexity of phagocyte recognition of apoptotic cells and suggest considerable functional redundancy in molecular pathways that are used for phagocytic clearance, perhaps ensuring that clearance of apoptotic cells is not compromised by genetic mutations in any one pathway.

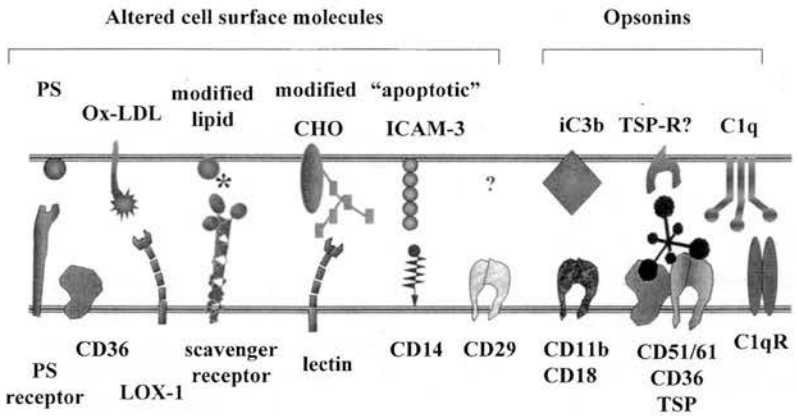

Fig 2. Cell surface molecules implicated in the uptake of apoptotic cells by phagocytes (see also Table I and text).

Table I. Phagocyte surface receptors that have been implicated in the recognition of apoptotic cells.

Surface molecule	Phagocyte	Apoptotic particle	Experimental evidence*
Lectin	Mouse peritoneal macrophage (Duvall *et al*, 1985) (Pradhan *et al*, 1997)	Thymocyte, lymphocyte	CL
Asialoglycoprotein receptor	Rat liver cell (Dini *et al*, 1992) Liver endothelial cell (Dini *et al*, 1995) Kupffer cell (Falasca *et al*, 1996)	Liver cell	CL
Mannose/fucose receptor	Human fibroblast (Hall *et al*, 1994)	Neutrophil	CL
CD36	Human THP-1 macrophage Mouse J774 macrophage (Tait & Smith, 1999)	Lipid microspheres	CL, AB
$\alpha v\beta 3$ (CD51/61)/thrombospondin/CD36	Human monocyte-derived macrophage (Savill *et al*, 1990, 1992)	Neutrophil	CL, AB, TF(CD36)
$\alpha v\beta 3$/thrombospondin	Human fibroblast (Hall *et al*, 1994)	Neutrophil	CL, AB
$\alpha v\beta 3$/thrombospondin/proteoglycans	Human glomerular mesangial cell (Hughes *et al*, 1997)	Neutrophil	CL, AB
$\alpha v\beta 3$	Mouse bone marrow-derived macrophage (Fadok *et al*, 1992a) J774 mouse macrophage (Pradhan *et al*, 1997)	Lymphocyte	CL, AB
	Human dendritic cell (Rubartelli *et al*, 1997)	Jurkat cell	CL, AB
CD29 ($\beta 1$)	Human monocytes U937 monocytes (Schwartz *et al*, 1999)	Endothelial cells	AB
Phosphatidylserine receptor	Mouse peritoneal macrophage (Fadok *et al*, 1992b) Stimulated bone marrow-derived macrophage (Fadok *et al*, 1992a)	Lymphocyte	CL
	Human PMA-stimulated THP-1 cell (Fadok *et al*, 1992a)	Lymphocyte	CL
	J774 mouse macrophage (Pradhan *et al*, 1997)	Lymphocyte	CL
	Rat vascular smooth muscle cell (Bennett *et al*, 1995)	Vascular smooth muscle cell	CL
	Rat Sertoli cell (Shiratsuchi *et al*, 1997)	Spermatogenic cell	CL
β_2-glycoprotein receptor	Mouse peritoneal macrophage (Balasubramanian *et al*, 1997)	Thymocytes	AB
Lox-1	Endothelial cells (Oka *et al*, 1998)	HL-60, Jurkat	CL, TF
OxLDL receptor	Mouse peritoneal macrophage (Chang *et al*, 1999)	Thymocytes	AB
Scavenger receptor	Mouse peritoneal macrophage Thymic macrophage (Platt *et al*, 1996)	Thymocyte	CL, AB, KO
CD14 (61D3 antigen)	Human monocyte-derived macrophage (Flora & Gregory, 1994; Devitt *et al*, 1998; Moffatt *et al*, 1999)	Lymphocyte, neutrophil	CL, AB, TF
	J774 mouse macrophage (Pradhan *et al*, 1997) Peritoneal macrophage (Pradhan *et al*, 1997)	Lymphocyte	AB
ATP binding cassette transporter (ABC1)	Mouse peritoneal macrophage (Luciani & Chimini, 1996)	Thymocyte	AB
Complement receptors CR3/CR4	Human retinoic acid-differentiated THP-1 cells (Takizawa *et al*, 1996)	Jurkat cell	AB
	Human monocytes-derived macrophage (Mevorach *et al*, 1998)	Thymocytes	AB, CL
C1q receptor	Cells in renal glomeruli (Botto *et al*, 1998)	Cells in renal glomeruli	KO

*CL, competitive ligand; AB, antibodies; KO, genetic deletion (knock-out); TF, transfection.

Interpretation of results from studies of phagocyte clearance of apoptotic cells has been complicated by a number of factors in addition to the existence of tissue-specific clearance pathways. The use of 'apoptotic' cell populations that may contain cells at different stages of apoptosis (Hebert *et al*, 1996) and variable numbers of necrotic cells (Vermes *et al*, 1997) represents an additional, frequently overlooked variable in the study of phagocytic clearance. Phagocytosis of 'late' apoptotic and necrotic cells may be mediated by different mechanisms with distinct functional consequences. Furthermore, the experimental approaches that have been used to determine the involvement of specific molecules in recognition may give rise to further ambiguity. Identification of the molecular pathways involved in phagocytosis of apoptotic cells has relied to a large extent on the use of monoclonal antibodies (mAbs) and competitive ligands. One of the potential problems of competitive ligands is that, if the affinity of binding is low, high concentrations of ligand are necessary to compete with surface presentation of multivalent ligands on the surface of apoptotic cells, with the possibility of non-specific effects. Intact mAb or bivalent $F(ab')_2$ fragments have the potential for cross-linking macrophage surface receptors (including Fc receptors via intact mAb) and the initiation of signalling cascades that may indirectly alter phagocyte function. For example, we have demonstrated previously that cross-linking CD44 with bivalent mAb augments phagocytosis of apoptotic cells (Hart *et al*, 1997). It is therefore possible that mAb may exert inhibitory effects without the recognized receptor necessarily being directly involved in the phagocytic process. It is notable that, with a few exceptions (Flora & Gregory, 1994), confirmation of direct receptor involvement using Fab′ fragments has not been made. Specific genetic deletion of putative apoptotic cell receptors has provided somewhat equivocal data, perhaps reflecting the functional redundancy of receptor usage discussed above. For example, absence of thrombospondin-1 expression resulted in pneumonia, together with marked changes in lung homeostasis, which might have reflected altered potential for clearance of apoptotic cells. However, these effects were largely attributed to the ability of thrombospondin-1 to activate the latent form of transforming growth factor (TGF)-β1 (Crawford *et al*, 1998). Similarly, deletion of the class A scavenger receptor had profound effects upon phagocytosis of apoptotic thymocytes *in vitro*, but did not alter the architecture of the thymus significantly (Platt *et al*, 1998b). However, the absence of a structural abnormality *in vivo* may be accounted for by a large functional reserve for apoptotic cell clearance capacity in the healthy, 'pathogen-free' animal.

Although gene transfection into cell types lacking putative apoptotic cell receptors provides evidence suggestive of direct involvement via a 'gain of function' (Ren *et al*, 1995), these experimental approaches may be limited by the requirement for phagocytic machinery that may not present in the transfected cell type. Recent studies examining the binding of phospholipid vesicles have demonstrated a direct role for CD36 binding to anionic phospholipids (Tait & Smith, 1999), and uptake of oxidized low-density lipoprotein (LDL) via scavenger receptor recognition of the lipid moiety rather than apoprotein B as described previously (Bird *et al*, 1999). Similar experimental approaches using binding of apoptotic cells to purified isolated cell surface receptors would provide definitive evidence for an apoptotic cell recognition receptor.

For each system described so far, it is also noteworthy that there is often considerable mismatch between observed levels of phagocytic responses in macrophage populations or transfected cells with the percentage of cells that express putative recognition molecules. For example, human monocytes express CD36 and CD14, yet they bind and ingest apoptotic cells very poorly, implying that these receptors may be necessary, but not sufficient, for efficient phagocytosis to occur. This suggestion is supported by the lack of complete inhibition of phagocytosis of apoptotic cells, even when multiple pathways are blocked, implying that further molecular pathways for removal of apoptotic cells remain to be identified.

One of the characteristics of apoptotic cell clearance mechanisms is the lack of induction of pro-inflammatory macrophage responses, which may be important for the silent clearance of apoptotic cells in normal physiological processes (Meagher *et al*, 1992; Savill *et al*, 1993; Fadok *et al*, 1998). In contrast, phagocyte clearance of 'post-apoptotic' or necrotic cells may trigger pro-inflammatory responses (Stern *et al*, 1996; Hughes *et al*, 1997). Fadok *et al* (1998) demonstrated that one potential mechanism for the inhibition of pro-inflammatory cytokine production after apoptotic cell phagocytosis might involve the production of TGF-β and prostaglandins to actively suppress pro-inflammatory mediator production. Active modulation of inflammatory or immune responsiveness by macrophages after apoptotic cell phagocytosis is further supported by preliminary findings that interleukin (IL)-10 may be released from monocytes in a CD36-dependent manner after interaction with apoptotic cells (Voll *et al*, 1997). Recent data suggest that macrophage phagocytosis of apoptotic cells may also lead to the release of soluble Fas-ligand with the potential to influence inflammatory cell survival (Brown & Savill, 1999). It is likely that progression of inflammatory responses and development of disease is profoundly influenced by the combination of these factors. Interestingly, analysis of interaction of type A scavenger receptors and CD36 with oxidized LDL (oxLDL) suggested that, in contrast to soluble oxLDL, surface-bound oxLDL induced macrophage secretion of H_2O_2. Inhibition studies indicated that CD36 might play a role in the promotion of secretion, suggesting that distinct CD36 ligands (apoptotic cells and oxLDL) might exert differential effects (Maxeiner *et al*, 1998). Similarly, CD14-mediated binding of lipopolysaccharide (LPS) has pro-inflammatory effects on macrophage behaviour, whereas apoptotic cell recognition via CD14 does not (Devitt *et al*, 1998). For many cellular molecular recognition processes (e.g. macrophage–T cell interactions involved in antigen presentation), co-operative receptor interactions are required to ensure appropriate responses in the interacting cell types. One possibility is that phagocytes use a number of pathways in parallel to ensure the rapid identification of membrane changes of apoptotic cells and that the repertoire of receptor engagement during apoptotic cell recognition determines the phagocyte responses initiated. One of the challenges for

future research on apoptotic cell recognition mechanisms is to define the pattern of utilization of distinct molecular pathways *in vivo* and the issue of functional redundancy or exclusivity using tissue-specific and temporal gene targeting strategies or double knock-out animals.

PHAGOCYTOSIS: GENERAL MECHANISMS

Understanding the basic cellular machinery required for apoptotic cell internalization and comparison with well-characterized [e.g. FcR- and complement receptor (CR)-mediated] phagocytic mechanisms may generate useful clues for understanding the underlying control processes involved and provide the basis for novel strategies for manipulation of apoptotic cell clearance for therapeutic gain.

The dissociation of two distinct phases (initial binding to specific receptors on the phagocyte surface and subsequent internalization) during phagocytosis of particles that have been opsonized with immunoglobulin (Ig) or complement has allowed detailed dissection of the pathways at the molecular level. Binding of opsonized ligands via FcR or CR occurs in the absence of intact microfilaments or microtubules (Newman *et al*, 1991; Caron & Hall, 1998). In contrast, attachment of apoptotic neutrophils appears to be both energy dependent and requires intact cytoskeletal elements. Little or no binding of apoptotic cells to human macrophages is observed at 4°C or when cytoskeletal elements are disrupted (our unpublished observations). These findings contrast with studies of scavenger receptor function, which show temperature-insensitive binding of lipid microspheres (Bird *et al*, 1999).

Particle internalization requires microfilament integrity in all three forms of phagocytosis. Fc phagocytosis is mediated by lamellipodia extension around the particle in a 'zipper-like' fashion (Griffin *et al*, 1975), and blockade of surface receptors after particle attachment inhibited internalization.

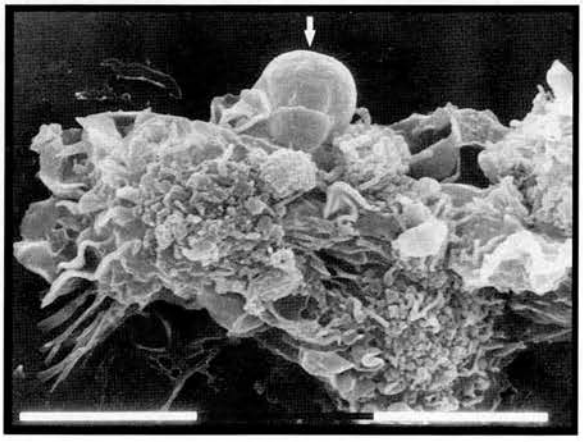

Fig 3. Scanning electron micrograph of the interaction between a human monocyte-derived macrophage and an apoptotic neutrophil *in vitro*. The apoptotic neutrophil has been bound to the ruffled membrane of the macrophage, and membrane projections can be seen extending around the apoptotic cell (arrowed). Bar = 10 μm.

Consistent with this model, inhibition of pseudopod extension by the PI-3 kinase inhibitor, wortmannin, prevented phagosome closure (Cox *et al*, 1999). CR1- and CR3-mediated ingestion also requires intact microfilaments but, in contrast to FcR-mediated internalization, bound complement-opsonized particles appear to 'sink' into the phagocyte surface, suggesting that internalization may occur via a 'modified zippering' mechanism involving the formation of a phagocytic pit (Kaplan, 1977). Further differences were revealed after stabilization of microtubules by elevation of cGMP, which enhances CR-mediated phagocytosis (Newman *et al*, 1991). Examination of macrophages ingesting apoptotic cells by scanning electron microscopy suggests that apoptotic cell uptake may be similar to the zippering mechanism observed for FcR-mediated phagocytosis (Fig 3). In support of this assertion, stabilization of microtubules via elevation of cGMP did not affect phagocytosis of apoptotic neutrophils, whereas microfilament disruption after cAMP elevation agents prevented ingestion (Rossi *et al*, 1998).

Ultrastructural identification of differences in phagocytic uptake is supported by recent molecular analysis of the recruitment of cytoskeletal elements to nascent phagosomes and regulation of cytoskeletal integrity by the Rho family of GTPases. These findings suggest intriguing parallels with the recruitment of proteins to nascent phagosomes and the formation of actin-rich podosomes containing talin and tyrosine-phosphorylated proteins that have been described during macrophage adherence (Marchisio *et al*, 1987). During FcR-mediated phagocytosis, cytoskeletal proteins (actin, talin, paxillin and α-actinin) and tyrosine-phosphorylated proteins are enriched near to the phagosome membrane in a diffuse manner, and phagocytosis is sensitive to tyrosine kinase inhibitors (Allen & Aderem, 1996). In contrast, during CR-mediated phagocytosis (which is insensitive to tyrosine kinase inhibition), discrete foci of these proteins are distributed over the phagosome surface. Consistent with the requirement for cytoskeletal elements described above, manipulation of Rho family GTPases does not alter FcR- or CR-mediated binding of particles. However, inhibition of formation of lamellipodial and filopodial membrane extensions by microinjection of dominant negative forms of Rho family GTPases, Rac and Cdc42, inhibits FcR-mediated phagocytosis (Caron & Hall, 1998). In addition, Rac and Cdc42 control membrane ruffling in macrophages, causing cell rounding and loss of integrin-containing focal complexes with co-localized vinculin, paxillin and FAK (Allen *et al*, 1997). In contrast, injection of the Rho inhibitor C3 transferase into macrophages inhibited the assembly of mechanotransducing actomyosin filaments and prevented CR-mediated phagocytosis (Allen *et al*, 1997; Caron & Hall, 1998). Distinct effects of Rho on cytoskeletal organization and adhesion were also observed with cell rounding after the injection of constitutively active Rho into macrophages, whereas inhibition of Rho led to loss of the presence of fine actin cables (Allen *et al*, 1997). These data demonstrate that there are distinct molecular mechanisms for phagocytosis via FcR and CR (termed type I and type II respectively), which may account for the observed differences in pro-inflammatory

mediator production after particle internalization (Wright & Silverstein, 1983; Yamamoto & Johnston, 1984; Aderem *et al*, 1985; Scholl *et al*, 1992; Debets *et al*, 1998).

As yet, there has been no direct demonstration of a role for Rho or Rac in apoptotic cell uptake. However, recent studies of *C. elegans* mutants that display defective phagocytic removal of cellular 'corpses' have characterized a number of genes involved in this process (Liu & Hengartner, 1998; Wu & Horvitz, 1998a,b), which suggest an important role for cytoskeletal-associated proteins and regulators in apoptotic cell uptake. The ced-6 gene product has sequences similar to phosphotyrosine-binding domains, and it may therefore act as an adaptor in the signalling pathways engaged after recognition and binding of apoptotic cells (Liu & Hengartner, 1998). Our preliminary data indicate that there is an absolute requirement for tyrosine kinase activity in apoptotic cell uptake, in keeping with the involvement of tyrosine phosphorylation and ultrastructural evidence for a 'zippering'-like mechanism (Fig 3).

The protein encoded for by ced-5, a gene that is required for the orderly engulfment of cell corpses in *C. elegans*, shows homology to the human CRK-binding protein DOCK180, which has been implicated in cytoskeletal function and extension of cell surfaces (Hasegawa *et al*, 1996; Wu & Horvitz, 1998a). We would suggest that phagocytosis of apoptotic cells shows characteristics that distinguish it from those involved in the clearance of opsonized particles, perhaps defining a novel 'type III' phagocytic mechanism. Further analysis of the basic phagocytic processes involved in the uptake of apoptotic cells will be required to identify the underlying control mechanisms.

REGULATION OF PHAGOCYTOSIS OF APOPTOTIC CELLS

Macrophage capacity for apoptotic cell phagocytosis is likely to be closely regulated in order that the tissue load of apoptotic cells is matched by appropriate clearance activity. Macrophage capacity for phagocytosis of apoptotic cells is influenced by soluble mediators, such as cytokines (Ren & Savill, 1995), prostaglandins (Rossi *et al*, 1998) and glucocorticoids (Lui *et al*, 1999), and by adhesive interactions with the extracellular matrix (McCutcheon *et al*, 1998). After binding of ligand to cell surface receptors, intracellular signals are transduced that may influence the capacity of the macrophage to phagocytose apoptotic cells (Fig 4). The potentially important ligand–receptor interactions and intracellular signalling pathways that are involved in the regulation of phagocytosis of apoptotic cells are beginning to be identified. Furthermore, as mentioned previously, analysis of the proteins encoded by engulfment genes in *C. elegans* has provided additional clues relating to the role of intracellular signalling pathways and the cytoskeleton in the phagocytosis of apoptotic cells in mammals.

SOLUBLE MEDIATORS

Co-ordination of cellular behaviour during the inflammatory response involves a complex interplay of cytokines. It is perhaps not surprising that, in addition to the modulation of apoptotic pathways in leucocytes (Lee *et al*, 1993), incubation of human monocyte-derived macrophages with granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-1β, interferon (IFN)-γ, tumour necrosis factor (TNF)-α or TGF-β1 for 4 h was found to augment macrophage phagocytosis of apoptotic neutrophils significantly (Ren & Savill, 1995). However, in rodent models, IFN-γ appears to exert inhibitory effects upon the uptake of apoptotic neutrophils, suggesting possible species differences (Erwig *et al*, 1999). More recently, we have identified a novel role for glucocorticoids in 'reprogramming' the phagocyte for enhanced recognition and phagocytosis (Lui *et al*, 1999). One implication of these findings is that increased capacity for disposal of apoptotic cells may contribute to the powerful anti-inflammatory effects of glucocorticoids.

In contrast to the relatively slow effects of cytokines and glucocorticoids, transient elevation of cAMP and activation of protein kinase A after exposure to inflammatory mediators and pharmacological agents that stimulate adenylate cyclase (e.g. prostaglandins) rapidly and specifically inhibit macrophage phagocytosis of apoptotic neutrophils (Rossi

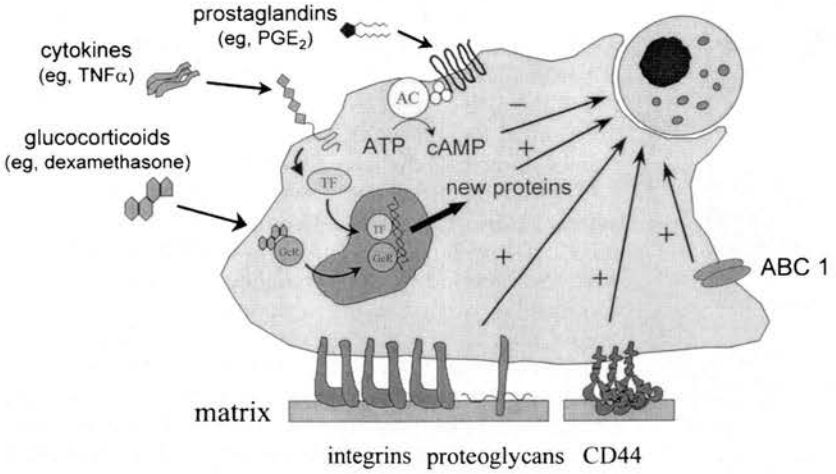

Fig 4. Regulation of macrophage phagocytic potential by soluble mediators, surface receptors and the concomitant intracellular signalling pathways (AC, adenylate cyclase; ABC1, ATP binding cassette transporter 1; GcR, glucocorticoid receptor; TF, transcription factor).

et al, 1998). Inhibition of macrophage phagocytic capacity for apoptotic cells persisted long after the intracellular cAMP concentration had fallen back to basal levels, implying that secondary signalling pathways had been initiated that ultimately determined phagocytic responses. Furthermore, activation of protein kinase C by phorbol esters enhanced macrophage phagocytosis of apoptotic neutrophils *in vitro*, while inhibition of tyrosine kinases with genestein had an inhibitory effect (unpublished observations). Together, these observations suggest that macrophage phagocytic capacity in the tissue may be dynamically 'fine-tuned' by the balance between the activity of different protein kinases.

ADHESION MODULATION AND PHAGOCYTOSIS OF APOPTOTIC CELLS

Phagocytosis shares mechanistic links with adhesion, spreading and migration, processes that require co-ordinated cytoskeletal reorganization, suggesting that regulatory factors may also have a role in the control of phagocytosis of apoptotic cells. Inhibition of apoptotic cell uptake after elevation of intracellular cAMP was associated with redistribution of cytoskeletal proteins, including actin, talin and paxillin, and profound morphological alterations (Rossi *et al*, 1998). In contrast, ligation of cell surface adhesion receptors by extracellular matrix (ECM) molecules leads to the assembly of cytoskeletal components and activation of intracellular signal transduction cascades (Giancotti, 1997), which, in concert with soluble chemical mediators, allows the behaviour of inflammatory cells to be co-ordinated (Nathan & Sporn, 1991). Remodelling of the ECM and temporal alterations in the matrix composition after injury or infection (Alitalo *et al*, 1980; Gailit & Clark, 1994) may therefore modulate the capacity for apoptotic cell phagocytosis during progression of the inflammatory response.

A precedent for the suggestion that adhesion receptor ligation may influence phagocyte capacity for clearance of apoptotic cells was provided by our demonstration that cross-linking of CD44 by bivalent antibodies rapidly (within minutes) augmented phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages (Hart *et al*, 1997). CD44 is a cell surface glycoprotein that has been proposed to bind multiple ligands in the ECM (including hyaluronan and fibronectin) and has been implicated in cytoskeletal organization and intracellular signalling (Lesley *et al*, 1993; Tsukita *et al*, 1994; Galandrini *et al*, 1996; Taher *et al*, 1996). Previously defined inhibitors of apoptotic cell recognition did not affect CD44-augmented phagocytosis of apoptotic neutrophils, suggesting either that several pathways were engaged together or that unique molecular recognition pathways were involved. This latter possibility was supported by the finding that CD44 mAb failed to augment phagocytosis of apoptotic lymphocytes.

Although there is evidence that $\beta1$ and $\beta3$ integrins may directly mediate apoptotic cell recognition (Savill *et al*, 1990; Schwartz *et al*, 1999), integrins may have a broader role in the control of phagocytic processes. Generation of 'outside-in' signals via integrins allows 'sensing' of the local microenvironment and may influence phagocytic function. In support of this suggestion, we have shown recently that adhesion of human monocyte-derived macrophages to fibronectin substantially augmented phagocytosis of apoptotic neutrophils (McCutcheon *et al*, 1998), an effect that was partially dependent on macrophage $\beta1$ integrins. However, ligation of $\alpha_v\beta_3$, $\alpha_L\beta_2$ or $\alpha_6\beta_1$, after apoptotic cell uptake or with mAb, was reported to inhibit subsequent phagocytosis when macrophages were rechallenged with apoptotic cells 48 h later (Erwig *et al*, 1999). Association of integrins with phosphatidylinositol 3-kinase (PI3K) (Lakkakorpi *et al*, 1997) or induction of tyrosine phosphorylation and activation of focal adhesion kinase-related molecule Pyk2 (Duong *et al*, 1998) after integrin-mediated binding of osteoclasts to matrix components illustrates how integrins might exert indirect effects.

It is interesting to note that other molecules implicated in apoptotic cell recognition have well-defined roles in adhesion processes. The macrophage scavenger receptor has a role in divalent cation-independent adhesion to serum-coated cell culture plastic (Fraser *et al*, 1993), and certain anti-CD36 mAbs may mimic many of the anti-adhesive effects of the ECM molecule thrombospondin (Dawson *et al*, 1997). Interestingly, CD36 may associate with tyrosine kinases Fyn, Yes and Lyn (Huang *et al*, 1991) and link directly with signal transduction cascades. Furthermore, ligation of cell surface molecules from diverse receptor families [e.g. urokinase-plasminogen activator receptor (CD87), integrin-associated protein (CD47), CD14, CD16, CD66 and CD31; Petty & Todd, 1996; Stocks *et al*, 1996] act to regulate adhesion (Klemke *et al*, 1998; Schaffner-Reckinger *et al*, 1998). Further investigation into the role of indirect effects (e.g. via adhesion modulation) is required in order to understand the regulation of apoptotic cell phagocytosis. One of the challenges for future studies is to distinguish receptors that may act as accessory molecules (in a manner analogous to (CD11a/CD18) LFA-1 and ICAM-1 (CD54) in antigen presentation via the T-cell receptor) from those that play a direct role in the recognition of apoptotic cells.

APOPTOTIC CELL LIGANDS

The final level of complexity concerning regulation of apoptotic cell uptake is the role of apoptotic cell ligands. Although it has been widely assumed that phagocytes are able to recognize alterations in the plasma membrane that distinguish apoptotic cells from their viable neighbours, it remains possible that phagocytes respond to a loss of signals that characterize interactions between viable cells. For example, in the absence of 'viable cell' signals, 'non-specific' phagocytosis mechanisms involved in the internalization of inert particles, such as latex microspheres or aldehyde-fixed cells, may be engaged. However, as discussed above, in contrast to apoptotic cell phagocytosis, uptake of inert particles elicits pro-inflammatory cytokine release in some phagocyte populations (Meagher *et al*, 1992). These data lend support to the concept that phagocytes have the capacity for recognition of determinants specific for apoptotic cells.

While a number of distinct phagocyte receptors involved in the recognition and subsequent internalization of dying

cells have been identified, the determinants on the surface of the apoptotic cell that signal phagocyte recognition and removal remain poorly characterized. For the erythrocyte (which has an average lifespan of 120 days in the healthy adult human), post-translational modification of the anion transport protein band 3 may lead to binding of endogenous IgG autoantibodies and subsequent Fc receptor-mediated phagocytosis (Kay, 1981; Kay *et al*, 1996). In parallel, non-enzymatic glycosylation of surface proteins to form advanced glycosylation end-products (AGE) may allow the engagement of specific receptors (e.g. the immunoglobulin superfamily member RAGE; Schmidt *et al*, 1994) that trigger clearance. However, the kinetics of appearance of AGE on senescent cells is likely to be too slow to serve as a signal for apoptotic cell recognition by phagocytes. In addition, the patterns of inhibition of macrophage phagocytosis of apoptotic neutrophils using polyanionic ligands indicated that AGE and AGE receptors were unlikely to participate (Savill *et al*, 1989).

Recent data indicate that rapid redistribution of aminophospholipids, including PS (Martin *et al*, 1995) and phosphatidylethanolamine (Emoto *et al*, 1997), may represent a general feature of apoptosis in many cell types. In viable cells, PS is confined to the cytoplasmic face of the plasma membrane by the action of a phospholipid translocase and is specifically relocated (possibly via specific phospholipid scramblases) to the outer surface of the membrane during apoptosis (Verhoven *et al*, 1995). PS exposure has been implicated in the recognition of apoptotic leucocytes by mouse inflammatory macrophages, but not by human monocyte-derived macrophages, *in vitro* (Fadok *et al*, 1992b). Binding of the serum β_2-glycoprotein I to exposed PS residues on apoptotic thymocytes and lipid symmetric red blood cell ghosts may 'mark' them for uptake by both thioglycollate-elicited mouse peritoneal macrophages (Balasubramanian *et al*, 1997) and the human THP-1 macrophage cell line (Balasubramanian & Schroit, 1998). Studies using the monocyte cell line THP-1 suggest that CD36 may act as a receptor for phosphatidylserine, with little or no specificity for other anionic phospholipids (phosphatidylinositol and phosphatidylglycerol). This activity appeared to involve sites in the receptor distinct from those involved in binding collagen or thrombospondin (Tait & Smith, 1999). In addition, anti-oxLDL autoantibodies cloned from Apo-E-deficient mice specifically bound to apoptotic aortic endothelial cells and prevented phagocytosis by mouse peritoneal macrophages (Chang *et al*, 1999). It is therefore possible that scavenger receptor-mediated recognition of oxidized phospholipids and oxidized phospholipid protein complexes may provide a specific signal for phagocyte binding and recognition of damaged or apoptotic cells.

It has been reported recently that complement components C1q and iC3b bind to the surface of certain apoptotic cell types, facilitating their uptake by macrophage C1q receptors or CR3/CR4 (Takizawa *et al*, 1996; Korb & Ahearn, 1997; Mevorach *et al*, 1998). These findings contrast with previous reports that CR3 was not involved in the recognition of apoptotic neutrophils by human monocyte-derived macrophages (Savill *et al*, 1989). Interestingly, cells in the late stages of apoptosis or blebs derived from apoptotic cells have been shown to contain self-antigens (Gilligan *et al*, 1996; Kalden, 1997), which are targets for autoantibodies found in autoimmune disease. One interpretation of these findings is that failure of tissue phagocytes to clear apoptotic cells may result in the development of autoimmune responses. Susceptibility of patients with C1q deficiency to the autoimmune disease systemic lupus erythematosus may be partly explained by the excess of free non-ingested apoptotic cells in the inflamed glomeruli seen in mice deficient in C1q (Botto *et al*, 1998).

Other molecular changes in the membrane of apoptotic cells have been studied using mAbs and lectins as probes. Early studies indicated that cellular microelectrophoretic mobility was reduced on apoptotic mouse thymocytes, suggestive of changes in the net negative charge of cell membranes (Morris *et al*, 1984). Reduced binding of lectins and mAbs observed in flow cytometric analysis corresponded closely with the degree of reduction in membrane surface area after apoptosis (Morris *et al*, 1984). It has been reported that high levels of mannose, *N*-acetylgalactosamine and galactose can be detected on apoptotic human peripheral blood lymphocytes using fluoresceinated lectins (Dini *et al*, 1992). However, flow cytometric analysis of binding of a panel of conjugated lectins to apoptotic neutrophils suggests that exposure of these carbohydrate moieties may not be a general feature of apoptosis on all cell types (unpublished observations). Screening of a large panel of mAbs revealed that blockade of certain epitopes of ICAM-3 (CD50) on apoptotic B cells was found to inhibit their phagocytosis by macrophages. These epitopes have been suggested as being associated with apoptotic cell-specific modifications of ICAM-3 recognized by macrophage CD14 (Gregory *et al*, 1998; Moffatt *et al*, 1999). There is evidence that specific changes in surface protein expression accompany apoptosis (Dransfield *et al*, 1994, 1995), implying that mechanisms for the maintenance of receptor numbers are compromised during programmed cell death. Neutrophil apoptosis is associated with a marked downregulation of FcγRIII (CD16) (Dransfield *et al*, 1994), leukosialin (CD43) and L-selectin (CD62L) (Dransfield *et al*, 1995), which may provide a mechanism for functionally isolating apoptotic neutrophils from potentially pro-inflammatory signals. As these receptors are shed by proteolytic mechanisms during activation, one possibility is that there is activation of surface-associated proteases during apoptosis or, alternatively, release of functional constraints upon protease activity by as yet undefined mechanisms. Proteolytic shedding of surface receptors during apoptosis has been observed in myeloid cell lines (Brown *et al*, 1996), but whether this represents a fundamental feature of apoptosis in different cell types remains to be determined. One possibility is that the presence of proteolytic 'stubs' of shed receptors could contribute to a 'signal' for phagocytosis.

CONCLUSION

Phagocytosis of apoptotic cells is a vital process in cellular homeostasis in a wide range of tissues, but especially so in the inflammatory response in which apoptotic granulocytes

have the potential to inflict tissue damage if they are not removed quickly and efficiently. Further work is required to identify cell surface molecules present on apoptotic cells that may trigger phagocyte recognition. Future research must resolve apparent differences in receptor usage in experimental models of apoptotic cell clearance in different species using different phagocyte and apoptotic cell populations. In addition, the role of macrophage receptors that may act as 'accessory' molecules that indirectly influence the recognition process needs to be defined. We would speculate that modulation of macrophage phagocytic capacity by a variety of cytokines, prostanoids, hormones, matrix molecules and possibly neighbouring cells may allow the tissue load of apoptotic cells to be matched by appropriate phagocyte clearance capacity, thereby minimizing release of histotoxic apoptotic cell contents. In contrast, phagocyte clearance capacity may be exceeded in pathological states. We suggest that therapeutic induction of cellular apoptosis to ameliorate tissue damage in inflammatory diseases, such as ARDS or rheumatoid arthritis, will require concurrent augmentation of phagocyte clearance capacity. Identification of the surface receptors involved in phagocyte recognition of apoptotic cells and definition of the contribution of regulatory influences *in vivo* will be important for the development of future therapeutic strategies for the treatment of inflammatory, allergic and autoimmune diseases.

ACKNOWLEDGMENTS

This work was supported by a University of Edinburgh Faculty of Medicine fellowship (to S.P.H.), the Wellcome Trust (K.M.G.) and the Medical Research Council.

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Keywords: apoptosis, phagocytosis, macrophage, inflammation, regulation.

Glucocorticoid Augmentation of Macrophage Capacity for Phagocytosis of Apoptotic Cells Is Associated with Reduced p130Cas Expression, Loss of Paxillin/pyk2 Phosphorylation, and High Levels of Active Rac[1]

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Phagocytic clearance of apoptotic granulocytes has a pivotal role in determining an inflammatory outcome, resolution or progression to a chronic state associated with development of fibrotic repair mechanisms, and/or autoimmune responses. In this study, we describe reprogramming of monocyte to macrophage differentiation by glucocorticoids, resulting in a marked augmentation of their capacity for phagocytosis of apoptotic neutrophils. This monocyte/macrophage phenotype was characterized by decreased phosphorylation, and therefore recruitment of paxillin and pyk2 to focal contacts and a down-regulation of p130Cas, a key adaptor molecule in integrin adhesion signaling. Glucocorticoid-treated cells also displayed higher levels of active Rac and cytoskeletal activity, which were mirrored by increases in phagocytic capability for apoptotic neutrophils. We propose that changes in the capacity for reorganization of cytoskeletal elements induced by glucocorticoids are essential for efficient phagocytic uptake of apoptotic cells. *The Journal of Immunology,* **2001, 167: 976–986.**

The acute inflammatory response provides a defense mechanism against microbial infection or tissue injury involving the rapid and coordinated recruitment of granulocytes and other inflammatory cells in response to chemokines and other inflammatory mediators. This response is normally self-resolving, but the pathogenesis of a number of diseases such as asthma, emphysema, and rheumatoid arthritis is characterized by a persistent accumulation of inflammatory cells presumably as a result of failure of the natural resolution process (1–3). During normal inflammatory resolution, clearance of extravasated granulocytes requires the induction of apoptosis and concomitant uptake by macrophages (4). Apoptotic cell death, in contrast to necrosis, is associated with maintenance of cell membrane integrity (4) and down-regulation of granulocyte secretory function (5), thereby inhibiting potential exacerbation of the inflammatory response through the release of cytotoxic granule contents and proinflammatory cytokines. Additionally, macrophage ingestion of apoptotic cells, unlike phagocytosis of necrotic cells or opsonized particles, does not induce proinflammatory mediator production (6) and can functionally down-regulate cytokine release induced by LPS or opsonized cells (7). Failure to clear apoptotic cells may result in secondary necrosis with predictable consequences in terms of tissue injury and/or initiation of autoimmune processes when phagocyte clearance processes in vivo are overwhelmed by excess apoptotic cell load (8–10). Clearly, if induction of apoptotic pathways is to be considered as a potential therapy for cancer or inflammatory disease, a parallel strategy to maximize phagocytic clearance is likely to be required to avoid the deleterious consequences of necrotic cell death.

A number of cell surface molecules have been proposed to mediate the uptake of apoptotic cells; these include lectins (11), $\alpha_v\beta_3$ integrin/CD36/thrombospondin complex (12, 13), phosphatidylserine receptors (14), scavenger receptors (15), receptors for oxidized lipids (16), CD14 (17), CD29 (18), the ABC1 transporter (19), and receptors for complement components C3bi (CR3/CR4) (20) and C1q (21). The lack of complete inhibition of phagocytosis by soluble ligands or blocking mAb points to functional redundancy, and we have suggested that a more effective strategy for altering clearance of apoptotic cells would be manipulation of macrophage phagocytic potential (22). Although cytokines such as TNF, GM-CSF, TGF-β, or IL-1 may be used to augment phagocytic clearance (23), the effects have been small and the pleiotropic consequences of some of these agents may restrict their potential therapeutic value. Our previous studies have revealed that disruption of cytoskeletal and adhesion contacts in monocyte-derived macrophages by elevation of intracellular cAMP inhibits phagocytosis of apoptotic cells (24). In contrast, ligation of CD44 (25) or adhesion to fibronectin (26) results in a rapid and dramatic augmentation of apoptotic cell uptake. For example, CD44 induces a 400% increase in phagocytic index within a 30-min assay period. If similar augmentation of phagocytic activity were attainable in vivo, the potential for clearance of apoptotic cells over the course of an inflammatory response would be considerable. Recent genetic studies in *Caenorhabditis elegans* have further suggested a role for adhesion signaling in the control of phagocytosis. Ced-5, -2, and -10, members of a family of genes required for clearance of cellular corpses during development, are highly homologous to mammalian proteins DOCK180 (27) (myoblast city protein in *Drosophila*) (28), Crk, and Rac (29), involved in mediating integrin signaling in mammalian cells. Integrin ligation induces the

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Received for publication December 11, 2000. Accepted for publication May 4, 2001.

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[1] This work was supported by Medical Research Council Program Grant 9016491 (to C.H., A.G.R., K.R., and I.D.) and the Wellcome Trust (to K.M.G.).

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0022-1767/01/$02.00

formation of a multiprotein complex involving DOCK180 and Crk, the adapter protein p130Cas, and the guanine nucleotide exchange factor C3G (30–33). Assembly and membrane localization of this complex activates the GTPases Rac (34, 35) and Rho (36), inducing membrane ruffling and lamellipodia formation (37) required for spreading, adhesion, and cell migration. Ced-5 and ced-2 have been shown to interact and activate ced-10 GTPase activity in vitro (29), and ced-5-, -2-, and -10-deficient animals have defects in both cell migration and phagocytosis (27, 29).

Glucocorticoids represent a powerful antiinflammatory treatment due to their capacity for inhibition of inflammatory cell recruitment and down-regulation of production and responsiveness of cells to proinflammatory cytokines (38). We have recently described a novel glucocorticoid receptor-dependent promotion of macrophage capacity for phagocytosis of apoptotic cells following short-term exposure of macrophages to glucocorticoids (39). In the present study, we show that long-term exposure of monocytes to the synthetic glucocorticoid dexamethasone (DX)[3] reprograms monocyte differentiation toward a proresolution phenotype, exhibiting increased phagocytosis of apoptotic cells. Monocytes treated with DX represented a homogeneous cell population characterized by a more rounded appearance. Marked down-regulation of expression of p130Cas, which is required for integrin adhesion signaling through the DOCK180/Crk/C3G complex, together with reduced phosphorylation and recruitment of paxillin and pyk2 to sites of adhesion, may account for this phenotypic alteration. We therefore propose that the dramatic increase in phagocytic potential in DX-treated monocyte-derived macrophages results from changes in the capacity for adhesion-dependent reorganization of cytoskeletal elements that are then available for coordinated phagocytic uptake of apoptotic cells.

Materials and Methods

Abs and other reagents

Reagents were obtained from Sigma (Poole, U.K.), unless otherwise stated. Iscove's DMEM (IDMEM) was from Life Technologies (Paisley, U.K.). Dextran and Percoll were from Amersham Pharmacia Biotech (Buckingham, U.K.). DX was obtained from David Bull Laboratories (Warwick, U.K.). Primary Abs were from the following sources: p130Cas, paxillin, Pyk2, Rac, and RC-20 (anti-phosphotyrosine) mAb were from Transduction Laboratories (supplied by Becton Dickinson, Oxford, U.K.); CrkL and C3G rabbit polyclonal Ab were from Santa Cruz (supplied by Insight Biotechnology, Wembley, U.K.). mAb specific for CD44v3 (3G5), β1 (12G10), and class II (WR18) were from Serotec (Oxford, U.K.). Control mouse Igs (IgG1and IgG2a), BerMac3 (CD163) and rabbit Igs, and F(ab′)$_2$ goat anti-mouse Ig FITC and HRP conjugates were from Dako (Ely, U.K.). The following monoclonals were generously provided as gifts: 5A4 (CD44; G. Dougherty, University of California, San Francisco, CA), SMϕ and 15.2 (CD36 and CD54, respectively; N. Hogg, Imperial Cancer Research Fund (London, U.K.), 23C6 (CD51/61; M. Horton, UCL, London, U.K.), PM6/13 (CD61; M. Wilkinson, Wellcome Trust, London, U.K.), 3G8 (CD16; J. Unkeless, Mount Sinai Medical School, New York, NY), UCHM1 (CD14; P. Beverley, UCL), 61D3 (CD14; C. Gregory, Nottingham, U.K.). Secondary anti-rabbit HRP was from Amersham Pharmacia Biotech.

Cell isolation and culture

Mononuclear and polymorphonuclear leukocytes were isolated as previously described (25). In brief, erythrocytes were sedimented from freshly drawn peripheral blood, with 0.6% (w/v) dextran T500, followed by fractionation of leukocytes on a discontinuous Percoll gradient (prepared in Ca^{2+}/Mg^{2+}-free PBS with final concentrations of Percoll of 50, 63, and 73%) at 720 × *g* for 20 min. Mononuclear cells were aspirated from the 50/63 interface, and neutrophils from the 63/73% interface, and washed three times in PBS (without Ca^{2+}/Mg^{2+}) before culture. Neutrophils (resuspended at 4 × 10^6cells/ml in IDMEM containing 10% autologous serum) were cultured at 37°C in a 5% CO_2 atmosphere for 20 h in Falcon tissue culture flasks. Cultured populations were >50% apoptotic, as determined by morphological analysis and annexin V binding, and <5% propidium iodide positive. Mononuclear cells were plated at 4 × 10^6/ml in IDMEM and incubated for 30–60 min, at 37°C, 5% CO_2 after which nonadherent lymphocytes were removed by washing with HBSS (without Ca^{2+}/Mg^{2+}) and monocytes were cultured for a period of 5 days in IDMEM plus 10% autologous serum, ±1 μM DX.

Macrophage phagocytosis assay

Monocyte-derived macrophages cultured in 48-well tissue culture plates, as described above, were cultured in the presence or absence of DX, or 10 μM RU38486 for varying periods of time. For experiments using inhibitors of phagocytosis, macrophages were washed once, then incubated with phagocytosis inhibitors (at the concentrations described in figure legends) for 15 min before the phagocytosis assay. The macrophage monolayer was then overlaid with apoptotic neutrophils (washed and resuspended at a final concentration of 4 × 10^6/ml in IDMEM) and incubated at 37°C, 5% CO_2 for 20 min. Noningested neutrophils were removed by washing in IDMEM, and monolayers were then fixed in 2.5% glutaraldehyde. The percentage of phagocytosis of neutrophils stained for myeloperoxidase activity with 0.1 mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxide was quantified microscopically by counting at least 500 cells in randomly selected fields per well, and an average between the duplicate wells was calculated. Phagocytic index was calculated as: (average number of neutrophils phagocytosed per macrophage) × (% macrophages that had phagocytosed one or more neutrophil). Controls for inhibitors used were as follows: All mAb used have previously been shown to inhibit apoptotic cell phagocytosis and were used at concentrations that were deemed to be saturating by flow cytometry. The following inhibitors were found to be functionally active at concentrations used in this study. RGDS (integrin inhibition peptide) was shown to inhibit $\alpha_5\beta_1$ integrin-mediated adhesion of T lymphocytes to fibronectin. Phospho-L-serine was shown to inhibit binding of FITC annexin V to apoptotic neutrophils in flow cytometric analysis. Dextran sulfate inhibited uptake of acetylated low density lipoprotein by monocyte-derived macrophages. Other reagents (glyburide and glucosamine) were used at concentrations that have previously been shown to exert inhibitory effects.

Flow cytometry

Flow cytometry was performed essentially as described (25), with all incubations conducted on ice to prevent internalization of Ab. Macrophages were detached from tissue culture plastic using PBS containing 2 mM EDTA and 0.5% serum. After washing with ice-cold PBS containing 0.2% (w/v) BSA and 0.1% (w/v) sodium azide cells (10^5/assay) were preincubated for 10 min with 20% (v/v) normal rabbit serum to block nonspecific binding to FcγR. Cells were then incubated with saturating concentrations of mAb for 30 min, and washed twice in PBS containing 0.2% BSA and 0.1% sodium azide before incubation with FITC-conjugated F(ab′)$_2$ goat anti-mouse Ig (Dako) for 30 min, and washed twice more before analysis using either an EPICS Profile II (Beckman-Coulter, High Wycombe, U.K.) or a FACSCalibur (Becton Dickinson) flow cytometer.

Electron microscopy

Macrophages cultured on glass coverslips in the presence or absence of 1 μM DX for 5 days were fixed with 3% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 3 h, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. After dehydration in an ascending acetone series, and critical point drying with CO_2, samples were sputter coated with 20 nm gold/palladium and examined using a Phillips 505 scanning electron microscope.

Immunoprecipitation and Western blotting

Adherent macrophage cultures were washed with PBS containing 0.1 mM $NaVO_3$ plus protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), and were lysed by incubation with PBS containing 1% Nonidet P-40, 0.1 mM $NaVO_3$, and protease inhibitor cocktail, 10 min on ice. Membrane and nuclear material were removed by centrifugation at 14,000 × *g*, 4°C, 30 min. Lysates were precleared by incubation with protein A agarose-coupled rabbit anti-mouse IgG, 4°C, 30 min. The resulting lysates were tested for protein concentration using a detergent-compatible protein estimation kit (Pierce, Rockford, IL), and equilibrated to contain equivalent levels of protein. A total of 100 μl lysate (100–150 μg total protein) was incubated with 1 μg of either mouse IgG control, anti-paxillin, or pyk2 mAb, 4°C, 30 min, shaking. Immunoprecipitation was achieved by incubation for 30 min with protein A-coupled rabbit anti-

[3] Abbreviations used in this paper: DX, dexamethasone; IDMEM, Iscove's DMEM; PAK, p21-activated kinase.

mouse IgG (Sigma), and washed twice in TBS containing 0.1% Triton X-100, and once in 25 mM Tris, plus 0.05% SDS. Samples were resolved using a 9% reducing polyacrylamide gel and transferred electrophoretically (50 V for 1 h) onto nitrocellulose (Amersham Pharmacia Biotech). For detection of phosphotyrosine, membranes were blocked with TBS plus 0.05% Tween 20 (TBS-T) and all other blots with TBS-T plus 10% nonfat dried milk powder (w/v).

Assay for detection of activated Rac

Adherent macrophage cultures were lysed in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Boehringer Mannheim) plus 1 mM PMSF. Lysates were cleared of membrane and nuclear material by centrifugation, total protein was estimated, and levels were equilibrated as described for immunoprecipitation. A total of 20 μl lysate was removed for estimation of total Rac protein, and the remaining (~300 μg) was incubated with GST-p21-activated kinase (PAK) (cdc42 or Rac interacting binding domain) fusion protein coupled to Sepharose beads, 4°C, 1 h, shaking. Beads were washed four times in ice-cold Tris buffer (50 mM Tris (pH 7.2), 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton, protease inhibitor mixture, 1 mM PMSF), and the amount of active Rac bound to the PAK cdc or Rac interacting binding domain quantified by SDS PAGE and Western blotting, as described for immunoprecipitation.

RNA isolation and RT-PCR

Macrophages were washed once in ice-cold PBS, lysed, and RNA extracted using TRIzol (Life Technologies; protocol as manufacturer's instructions). RNA was DNase treated to remove genomic DNA for 1 h, 37°C, and the resulting RNA was used in RT-PCR reaction using Life Technologies One Step RT-PCR kit (protocol as manufacturer's instructions). Primers used (35 cycles, annealing 53°C) were as follows: DOCK180, 5′-GAGGCAGAGGAGACGAACAG, 3′-AAGCCGATTCG GTGTAGTTA; M-DOCK, 5′-TGCTGAAGTGGCGTATGAAG, 3′-CCTCGACCGAACAATGAACT; actin, 5′-CCACCAACTGGGACGA CATG, 3′-GTCTCAAACATGATCTGGGTCATC.

Results

Glucocorticoids augment phagocytosis of apoptotic cells in a time-dependent manner

Monocytes isolated from peripheral blood were cultured in vitro for up to 5 days in the presence of the synthetic glucocorticoid DX, and macrophage potential for phagocytosis of apoptotic cells was determined. Preliminary experiments revealed that macrophages treated with DX for >24 h were highly phagocytic, leading to some cell detachment during our standard assay (data not shown). We therefore reduced the assay time from 30 to 20 min to minimize cell loss and reveal differences between treated cells. DX treatment consistently increased monocyte-derived macrophage phagocytosis of apoptotic neutrophils in a manner that was related to the duration of treatment (Fig. 1*A*), and blocked by addition of the steroid receptor antagonist RU38486 (10 μM), indicating that a glucocorticoid receptor-dependent process mediated these effects (results not shown). In particular, we noted a striking effect of exposure of freshly isolated monocytes to DX for 120 h upon phagocytic potential. We therefore investigated whether a time window for steroid exposure early in maturation was sufficient to generate a highly phagocytic phenotype. Monocytes were incubated with DX for periods of 24 h (0–24, 24–48, 48–72, 72–96, and 96–120). To ensure that observed changes were the result of DX exposure exclusively during the stated time window, the steroid receptor antagonist RU38486 was added following removal of DX-containing media. The addition of the antagonist alone had no significant effect (data not shown). Augmentation of apoptotic cell phagocytosis was much less pronounced when DX was added later in the culture period, and that exposure of monocytes to DX within the first 24 h was sufficient to induce highly phagocytic macrophages (Fig. 1*B*). Comparison of phagocytic indices revealed that 5-day DX-treated monocytes/macrophages had a 9-fold higher capacity for apoptotic cell clearance in a 20-min assay (Fig. 1, *C–E*). Thus, glucocorticoids represent the most potent stimulus for augmentation of macrophage capacity for phagocytosis of apoptotic cells described to date. Moreover, glucocorticoid modulation of phagocytosis could have profound effects on clearance of apoptotic cells during the resolution of inflammation.

Glucocorticoids reprogram macrophage phenotype

Examination of macrophages by phase-contrast microscopy revealed that augmented phagocytic potential following 5-day treatment with DX was accompanied by distinct morphological changes consistent with the suggestion that DX influences in vitro differentiation of monocytes. Under our standard culture conditions (IDMEM containing 10% autologous serum) at 5 days, macrophages were composed of a heterogeneous population of cells with different morphologies: multinucleated giant cells, spread apparently motile cells with evidence of membrane ruffling, rounded less spread cells, and occasional dendritic-like cells (Fig. 2*A*). Microscopy-based quantitation revealed that large macrophages (>25 μm diameter) represented 29.5 ± 5.8% of cells present in 5-day monocyte/macrophage preparations (n = 4 separate experiments). Treatment of freshly isolated monocytes with DX for 5 days resulted in consistent morphological changes, with the absence of large multinucleated cell populations and reduced numbers of spread macrophages (large macrophages representing less than 2% of the cells present). In contrast, the proportion of rounded cells was increased (Fig. 2*B*). Further examination of macrophage morphology using scanning electron microscopy demonstrated that the rounded DX-treated cells were attached, with ruffled membranes and filopodial processes (Fig. 2*D*) when compared with untreated cells (Fig. 2*C*). An increase in the morphological homogeneity of DX-treated monocyte-derived macrophages was also reflected in the laser scatter properties of DX-treated cells and more uniform expression of a number of surface molecules, including CD14, when analyzed by flow cytometry (Fig. 2*E*). Analysis of the coefficient of variation for the fluorescence peaks (untreated vs DX-treated ± SEM) for CD14 (108 ± 14; 60 ± 5, n = 13), CD16 (96 ± 8; 62 ± 4, n = 11), HLA-DR (122 ± 11; 96 ± 6, n = 10), CD44 (99 ± 15; 66 ± 7, n = 6), and CD51 (81 ± 5; 53 ± 4, n = 11) confirmed this impression and would be consistent with reprogramming of monocyte differentiation by DX during in vitro culture.

Increased phagocytosis of apoptotic cells involves multiple phagocytic receptors

Flow cytometric analysis was further used to determine whether DX augmentation of phagocytosis was associated with increased expression of receptors previously implicated in the recognition process (see Ref. 22 for review and Table I). Although most receptors examined exhibited more uniform levels of surface expression, as described above, we did not observe changes in the percentages of positive cells following DX treatment. However, comparison of mean fluorescence intensity of binding revealed some consistent alterations in the levels of surface expression of certain receptors. HLA-DR and the macrophage differentiation markers FcγRIII (CD16) and BerMac (CD163) were expressed at slightly elevated levels on DX-treated macrophages (Table I). In contrast, we found significantly reduced expression of CD44, CD44v3, ICAM-1 (CD54), and integrin β_3 subunit (CD61) ($p <$ 0.05 using Student's t test). Binding of the CD36 mAb SMϕ was always found to be lower than that of the IVC7 CD36 mAb, even when both Abs are used at saturating concentrations, suggesting that SMϕ may recognize an epitope that is not present on all CD36 molecules. Interestingly, although overall levels of CD36 expression (IVC7 staining) were decreased with DX treatment, binding of the SMϕ mAb was not reduced. Together these results indicated

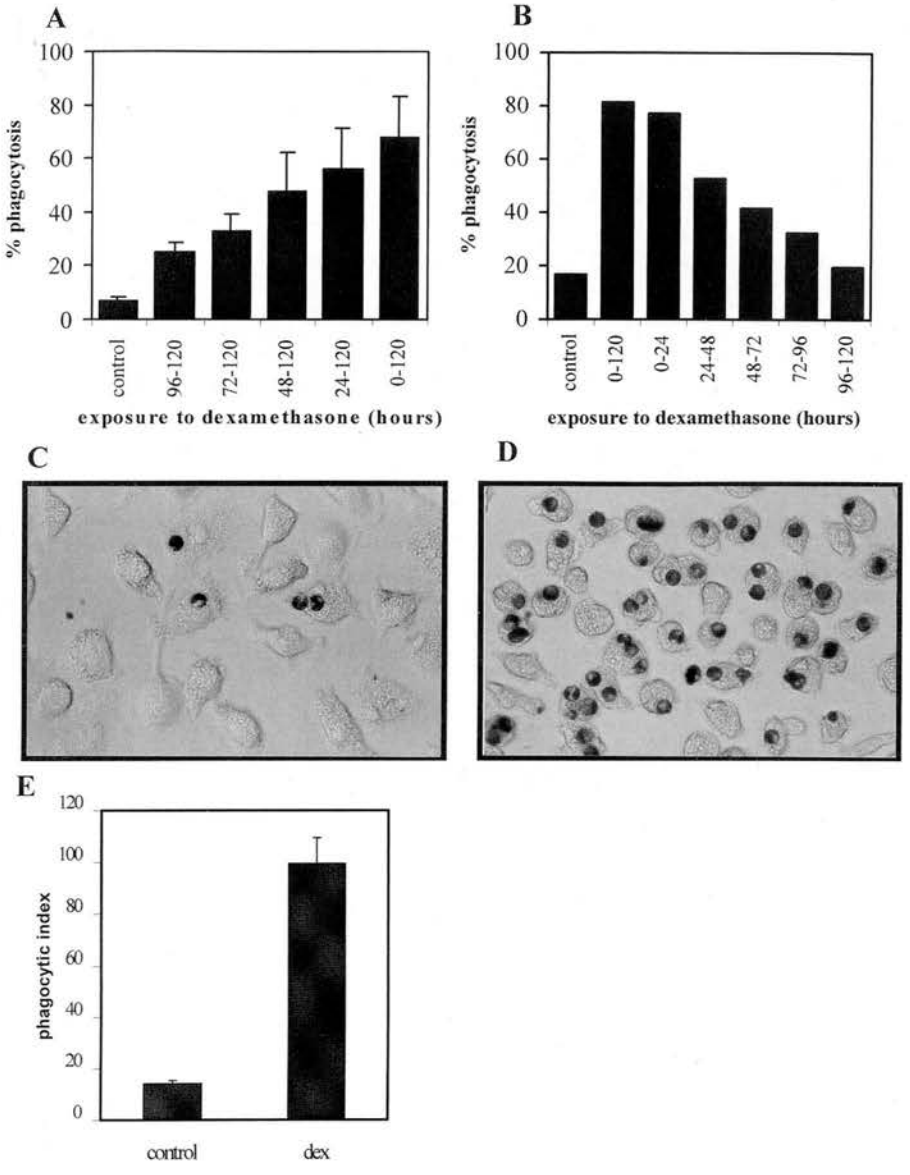

FIGURE 1. Effects of DX on macrophage phagocytosis of apoptotic neutrophils. Adherent peripheral blood monocyte-derived macrophages were cultured in the presence or absence of 1 μM DX for different times, as shown. The capacity for phagocytosis of apoptotic neutrophils was determined in a 20-min assay by microscopic visualization of myeloperoxidase activity. *A*, Monocyte-derived macrophages were incubated with 1 μM DX for the time periods shown during 5 days of culture before assessment of phagocytosis. Data shown as mean phagocytosis ± SEM for three separate experiments. *B*, Monocyte-derived macrophages were exposed to a 24-h pulse of 1 μM DX for the time periods shown during 5 days of culture, before assessment of phagocytosis. Data shown are one representative experiment of four that were performed. Representative photomicrographs showing untreated and 5-day DX-treated monocyte-derived macrophage capacity for phagocytosis are shown in *C* and *D*, respectively. Phagocytic index (calculated as (average number of neutrophils phagocytosed/macrophage) × (percentage of macrophages phagocytosing one or more neutrophils) for untreated and 5-day DX-treated monocyte-derived macrophages (values taken from mean of duplicates of a minimum of 500 cells ± SEM for five separate experiments) is shown in *E*.

that augmented phagocytic capacity was not associated with increased expression of putative apoptotic cell recognition receptors. However, since surface expression does not necessarily indicate the presence of functionally active receptors, we used specific mAb/soluble ligand inhibitors of apoptotic recognition pathways to define their contribution to DX-treated macrophage phagocytic activity. Inhibitors of CD36 (SMϕ mAb, 1:50 ascites) and $\alpha_v\beta_3$ (0.5 mM RGDS peptide) did not prevent phagocytosis of apoptotic neutrophils by DX-treated macrophages (Table I), suggesting that this pathway does not play a major role in DX-augmented phagocytosis. In addition, 10 mM glucosamine exhibited only partial inhibition of DX-augmented phagocytosis, further suggesting that integrin-mediated recognition is not the dominant pathway utilized. However, it should be noted that although we have validated that RGDS is functionally active in preventing $\alpha_5\beta_1$-mediated T cell adhesion to fibronectin, in our experimental system RGDS does not inhibit untreated macrophage phagocytosis either (percentage of phagocytosis control 28.5 ± 4%, RGDS 27.5 ± 4%; mean ± SEM, $n = 7$). Therefore, these data cannot be taken to indicate that treatment of monocytes with DX induces a switch to $\alpha_v\beta_3$-independent recognition of apoptotic cells. Inhibition of CD14 with the 61D3 mAb also failed to inhibit DX-treated macrophage phagocytosis of apoptotic neutrophils, suggesting that glucocorticoid up-regulated phagocytosis was not CD14 dependent (data not shown). We observed a small inhibitory effect of 2 mM phospho-L-serine upon DX-induced phagocytosis of apoptotic neutrophils, implying that the phosphatidylserine receptor does not play a major role. However, it is possible that phospho-L-serine is not the most effective inhibitor of phosphatidylserine receptor function. Phagocytosis was markedly inhibited in the presence of 500 μg/ml fucoidan (31% of control, $p < 0.01$), implicating scavenger receptor involvement. However, other scavenger receptor ligands, including dextran sulfate (Table I), only partially inhibited phagocytosis. These data suggest broader effects of fucoidan, which may also inhibit other carbohydrate recognition molecules (for example, selectins). One interpretation of these data is that DX augmentation may involve multiple receptor pathways acting cooperatively. Alternatively, DX might promote engagement of a novel receptor pathway to provide more efficient apoptotic cell recognition.

Glucocorticoids alter macrophage cytoskeletal organization

The distinct morphological appearance of DX-treated macrophages shown in Fig. 2 suggested that control of adhesion was

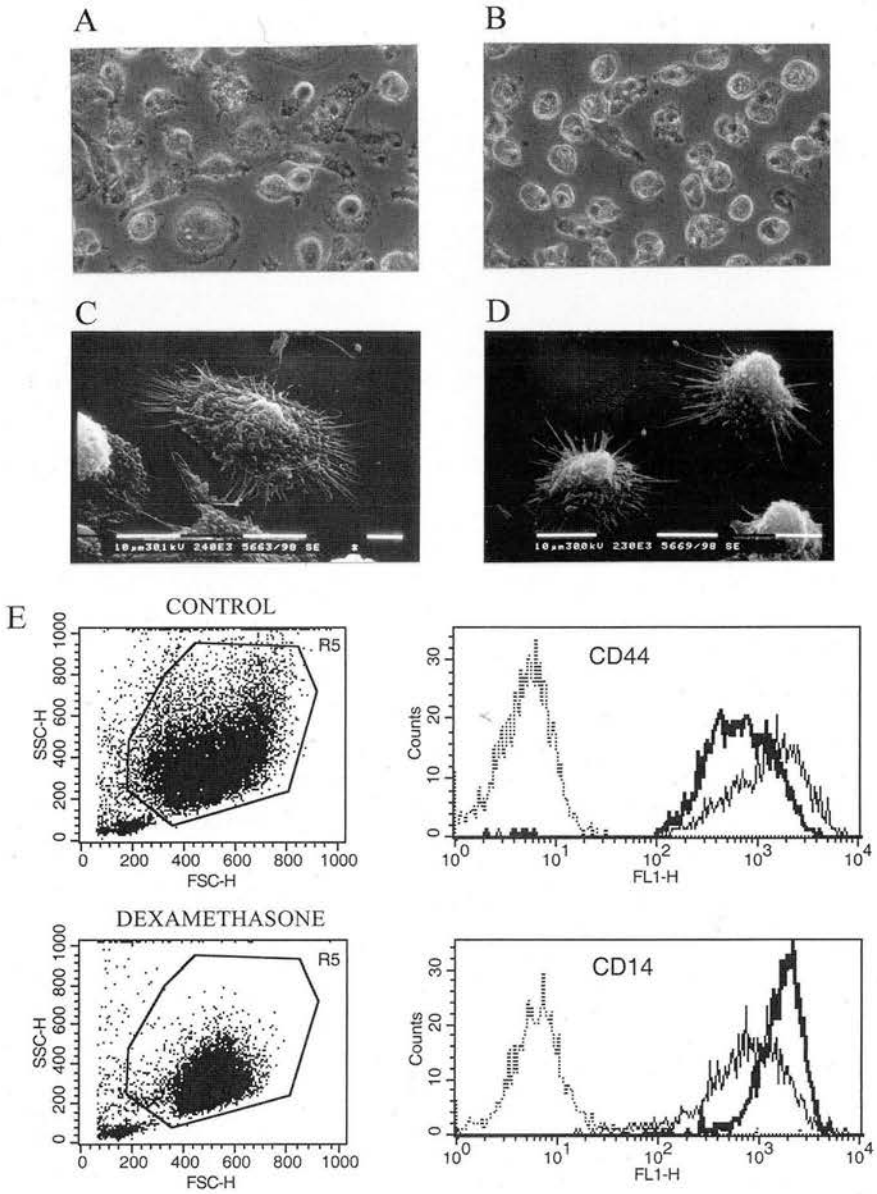

FIGURE 2. Effects of DX on macrophage phenotype. Adherent peripheral blood monocytes cultured for 5 days in the presence or absence of 1 μM DX on either tissue culture-treated plastic (*A* and *B*) or glass coverslips (*C* and *D*). Morphology of untreated (*A* and *C*), and DX-treated (*B* and *D*) was assessed by phase-contrast microscopy (*A* and *B*) ($\times$40 objective), and scanning electron microscopy (*C* and *D*) (bar, 10 μm). Note the uniform appearance of DX-treated cells by phase-contrast microscopy, which is also observed in electron micrographs. *E*, Monocyte-derived macrophage surface phenotype was examined by indirect immunofluorescence using control IgG1 mAb, CD44 mAb (5A4), or CD14 mAb (UCHM1) together with flow cytometry. The profiles (from one of at least four separate determinations) shown here illustrate the more homogeneous laser scatter properties of DX-treated macrophages. The fluorescence histograms (FL1-H) for cells within the gates shown illustrate the more uniform cell surface expression of CD14 and CD44 following DX treatment. Dotted lines show binding of relevant isotype controls; solid lines, mAb binding to untreated macrophages; and bold lines, mAb binding to DX-treated macrophages. FSC, Forward scatter; ssc, side scatter.

altered following DX treatment. In untreated cells, visualization of focal contacts within macrophages cultured for 5 days on glass slides showed punctate (podosome-like) staining of actin (Fig. 3*C*) with concentric association of talin (Ref. 24 and our unpublished data) and paxillin (Fig. 3*D*), probably representing sites of cell-substratum contact. Paxillin and other proteins associated with adhesion (vinculin and tyrosine-phosphorylated proteins (data not shown)) were also observed toward the cell periphery (see Fig. 3*D*), consistent with the adherent phenotype of macrophages. In contrast, DX-treated cells showed an absence of actin- and paxillin-containing podosomes (Fig. 3, *E* and *F*), suggesting an altered organization of adhesion structures.

Altered cytoskeletal protein phosphorylation and organization in DX-treated macrophages

Since paxillin recruitment to sites of adhesion is regulated by phosphorylation, we next examined levels of expression and the tyrosine phosphorylation status of paxillin and pyk2 by Western blot analysis of immunoprecipitated proteins. Although paxillin and pyk2 are still expressed at equivalent levels in DX-treated macrophages (not shown), phosphorylation was found to be reduced in adherent DX-treated macrophages when compared with untreated macrophages (Fig. 4). The observed decrease in phosphorylation of paxillin and pyk2 was consistent with the altered distribution of paxillin in macrophages and raised the possibility that DX treatment disrupted adhesion-dependent signaling. The p130Cas/Crk/DOCK180 complex is a major mediator of adhesion signaling. We therefore examined the levels of expression of CrkL, CrkII, and p130Cas by Western blot analysis, and since we were unable to reproducibly immunoblot DOCK180 (data not shown), we used RT-PCR to test for mRNA for DOCK180 and M-DOCK. Although we did not see changes in levels of CrkL (Fig. 5*A*) or CrkII (data not shown), p130Cas expression was markedly reduced in DX-treated macrophages (Fig. 5*B*). No differences in the levels of mRNA for DOCK180 or M-DOCK were noted in PCR analysis (Fig. 5*C*), although differences may be apparent at the level of protein. Despite these marked changes in cytoskeletal organization and phosphorylation, we were surprised to find that DX-treated cells were extremely active in formation of lamellipodia and cellular extensions necessary for phagocytosis in time lapse video

Table I. *Monocytes treated for 5 days with 1 μM DX, and the effects on surface receptor expression and apoptotic cell recognition pathways using previously defined inhibitors of phagocytosis*

	Expression		Function		
	mAb	% relative to control[a]	Inhibitor	% phagocytosis relative to control[a]	Ref.
$\alpha_v\beta_3$/CD36/thrombospondin					
α_v	13C2	98.7 ± 7.3			
β_3	Im6/13	82.7 ± 7.7			
$\alpha_v\beta_3$	23C6	83.6 ± 7.0	RGDS (active)	85.7 ± 5.7 (n = 7)	12, 13
			RADS (inactive)	101.3 ± 8.6 (n = 3)	
CD36	ICV7	58.1 ± 8.1			12, 13
	Smϕ	86.6 ± 20.0	Smϕ	76.6 ± 7.0 (n = 9)	
Charge sensitive			Glucosamine	63.8 ± 10.0 (n = 7)	
Pattern recognition receptors					
Mannose receptor			Mannan	81.5 ± 5.8 (n = 8)	57
CD14/ICAM-3	UCHM1	94.9 ± 14.4			17
CD44					
CD44 (all isoforms)	5A4	71.6 ± 9.0			25
CD44$_v$3	3G5	58.4 ± 6.4			
Phosphatidylserine receptor			Phospho-L-serine	80.8 ± 6.3 (n = 8)	14
"Scavenger" receptors					
SR-A			Dextran sulfate	67.9 ± 8.6 (n = 10)	15
CD163	BerMac3	189.9 ± 43.3			
Other molecules					
HLA-DR	WR18	123.6 ± 20.6	NA[b]	NA	
CD16	3G8	130.5 ± 11.5	NA	NA	

[a] "Control" receptor expression represents expression on 5-day DX-treated monocyte-derived macrophages relative to untreated 5-day monocyte-derived macrophages. "Control" phagocytosis represents phagocytosis of apoptotic neutrophils by 5-day DX-treated monocyte-derived macrophages in the absence of inhibitor.
[b] NA, Not available.

microscopy analysis (data not shown). To test whether the actin-regulatory machinery remained functional in DX-treated macrophages, we assessed the levels of activity of the Rho family GTPase Rac, which is involved in membrane ruffling and extension of cellular processes. Using pull-down assays with p21-activated kinase-GST agarose, we found that DX caused a marked increase in the amount of active Rac detectable within macrophage lysates (Fig. 5*D*). The pronounced morphological alteration observed in DX-treated cells may reflect reprogramming of the capacity for adhesion-dependent signal transduction via down-regulation of p130Cas, reduced paxillin, and *pyk2* phosphorylation, and failure to form podosome-like adhesion structures. However, DX-treated macrophages were found to have high levels of active Rac that might contribute to the increased capacity for cytoskeletal reorganization necessary for phagocytosis.

Discussion

In this study, we present evidence that a macrophage phenotype with augmented phagocytic potential for clearance of apoptotic cells is induced following exposure to DX. We believe that DX exerts distinct regulatory mechanisms upon macrophage behavior, depending upon the stage of monocyte maturation. In our previously published work, monocytes/macrophages were treated with DX from 96 to 120 h, leading to a relatively modest increase in phagocytic potential (39). In contrast with the morphological changes described in this work for cells treated with DX immediately following isolation, no gross changes in morphology are observed following exposure of 96-h monocytes/macrophages to DX (data not shown). Importantly, we demonstrate that treatment of monocytes with DX for the first 24 h of the 5-day culture period following isolation from peripheral blood is critical for induction of this phenotype, suggesting that glucocorticoids, acting via glucocorticoid receptors, have the potential to reprogram monocyte differentiation. In support of our suggestion, we observe that DX-treated monocytes mature into macrophages that exhibit a uniform morphological appearance consisting of smaller, more rounded cells with more homogeneous laser scatter properties in flow cytometric analysis. Although a number of macrophage surface receptors are expressed at slightly reduced levels in DX-treated cells when compared with untreated cells, the range of expression within the population was found to be less in DX-treated monocytes/macrophages, indicating that functional homogeneity is matched by cell surface phenotype. In addition, the specific increase in BerMac (CD163) expression following DX treatment lends further support to the suggestion of a reprogramming event. However, our analysis failed to reveal any single surface molecular change that would define a prophagocytic phenotype.

In preliminary experiments, we noted that highly phagocytic macrophages were prone to detach during washing following a 30-min assay. One speculation would be that internalization of plasma membrane during phagocytosis of apoptotic cells may compromise cellular adhesion. In addition, the marked functional alterations that we observe following glucocorticoid treatment of monocytes further illustrate a close association between control of monocyte/macrophage adhesion and clearance of apoptotic cells. Many of the receptors implicated in phagocytosis also have key roles in macrophage adhesion and migration (22). In this study, we demonstrate that augmentation of phagocytic potential is associated with decreased tyrosine phosphorylation of paxillin and *pyk2*, proteins that represent important components of adhesion contacts (for review, see Ref. 40), and decreased expression of p130Cas, a mediator of adhesion signaling (32). Immunofluorescence analysis revealed that adhesion structures containing localized paxillin and actin were altered in DX-treated macrophages. Reduced expression of p130Cas would be predicted to disrupt Crk/DOCK180

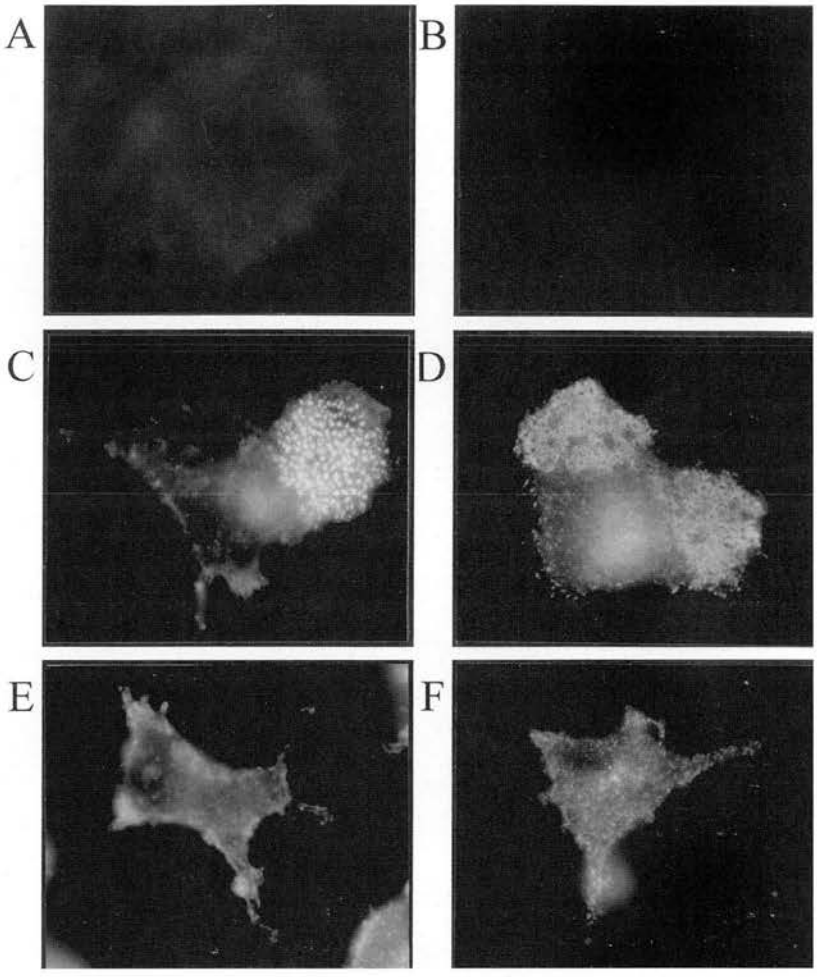

FIGURE 3. Effects of DX on localization of actin and paxillin in macrophages. Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1 μM DX. Localization of actin and paxillin was determined using either rhodamine phalloidin (*C* and *E*) or anti-paxillin mAb (*D* and *F*) together with fluorescence microscopy. Staining observed with control mAb is shown in *A* and *B*. These representative micrographs illustrate typical punctate actin staining of contact sites similar to podosomes in control macrophages (*C* and *D*) that are absent in DX-treated macrophages (*E* and *F*). Paxillin is also localized to smaller focal adhesion-like structures near the cell periphery in control macrophages that are less well defined in DX-treated macrophages.

complexes, which together with reduced phosphorylation of paxillin and pyk2 may have implications for control of the turnover of adhesion structures in macrophages (shown schematically in Fig. 6). Since decreased p130Cas expression in DX-treated macrophages is associated with augmentation of phagocytic capacity, specific recruitment of p130Cas to focal contacts following adhesion to matrix may mimic loss of p130Cas observed in DX-treated cells and influence the availability of other components to drive cytoskeletal reorganization necessary for phagocytosis. We have tried to define whether cytoskeletal components we have examined contribute directly to phagocytosis. However, these studies are made difficult by the morphological and functional heterogeneity of macrophage preparations. Furthermore, cytoskeletal elements present within internalized apoptotic neutrophils complicate interpretation of observed staining patterns. Both Crk and p130Cas have previously been shown to influence the capacity for actin reorganization in rat-1 fibroblast cells (41, 42); therefore, down-regulation of the central signaling molecule p130Cas is likely to have important implications for the control of adhesion and migration in DX-treated monocytes. We propose that the repertoire of adhesion receptors that are engaged on the macrophage surface might control phagocytic potential indirectly by releasing or sequestering key regulatory molecules such as p130Cas from focal adhesion complexes.

Time-lapse video microscopy reveals that despite the small rounded appearance of DX monocytes/macrophages, these cells remain extremely membrane active, rapidly extending and retracting cellular processes (data not shown). Increased levels of active Rac in DX-treated macrophages lend support to the suggestion that although recruitment of proteins such as paxillin to podosome adhesion-signaling complexes does not occur in the absence of p130Cas, Rac may still drive the extension and retraction of processes observed in DX-treated cells. One possibility is that other p130Cas-like adapters such as HEF1 and Efts/Sin (Ref. 32 and

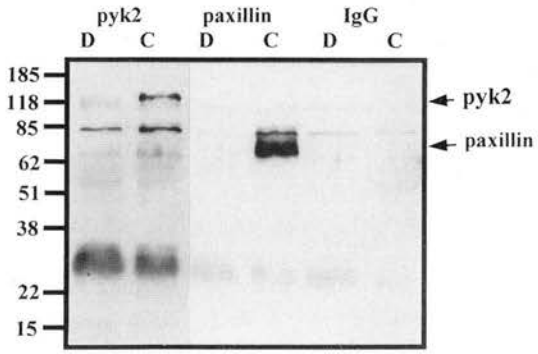

FIGURE 4. DX down-regulates phosphorylation of pyk2 and paxillin. Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1 μM DX. Pyk2 and paxillin were immunoprecipitated from cell lysates, and protein phosphorylation of immunoprecipitated proteins was analyzed by SDS-PAGE together with Western blotting using the anti-phosphotyrosine mAb RC-20 and ECL detection. In this gel, IgG denotes protein phosphorylation patterns associated with a nonbinding IgG1 control mAb. The band at ~85 kDa, present to some extent in all immunoprecipitates, probably represents a nonspecific component. C lanes are from untreated control macrophages, and D lanes are from DX-treated macrophages.

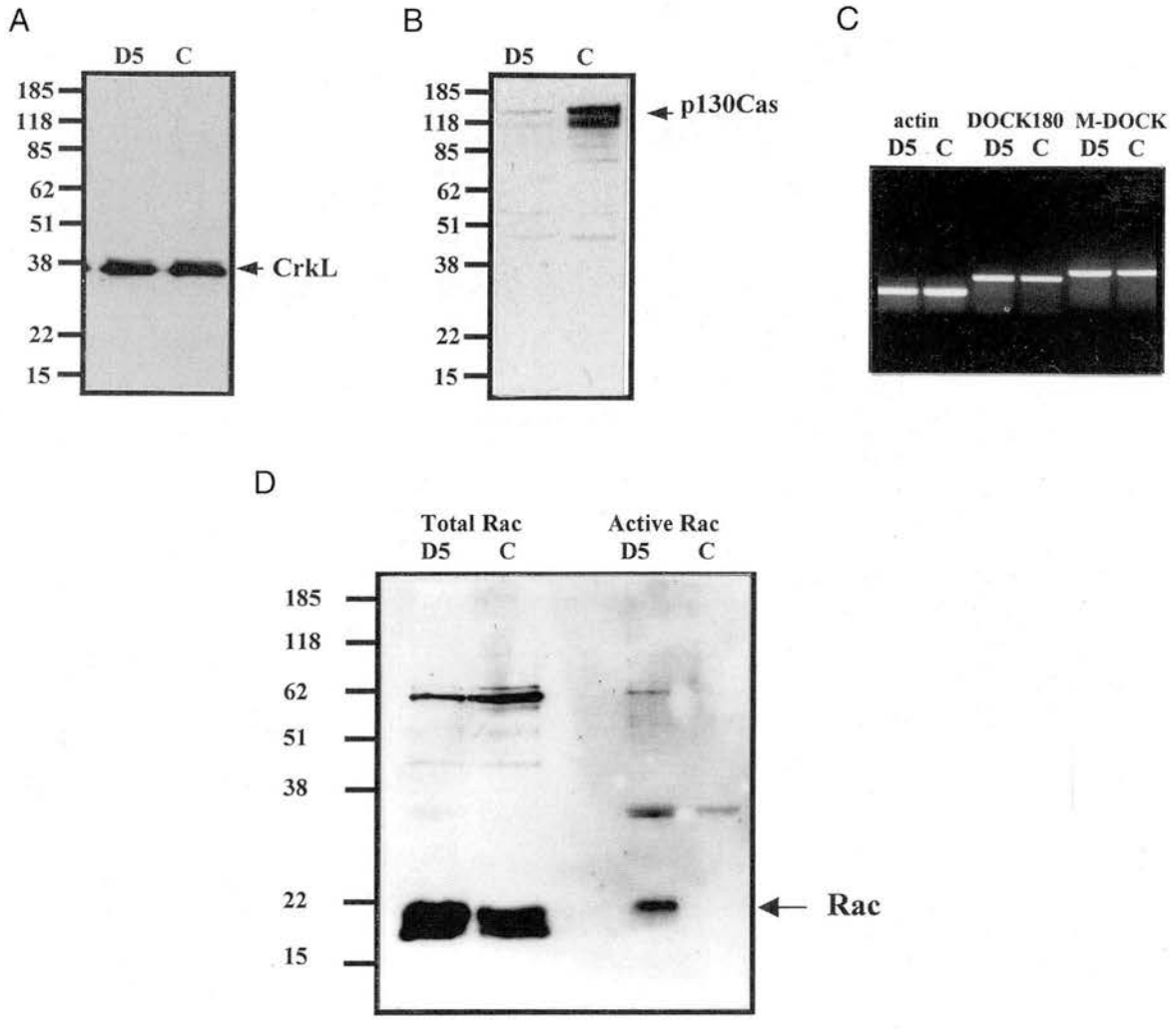

FIGURE 5. Specific down-regulation of p130Cas and increased activation of Rac following DX treatment of macrophages. Adherent peripheral blood monocyte-derived macrophages were cultured in the absence (C) or presence of 1 μM DX for 5 days (D5). Macrophage cell lysates were assessed for expression of *A*, CrkL (36 kDa) and *B*, p130Cas (130 kDa) by SDS-PAGE and Western blotting. *C*, Total RNA from untreated and DX-treated macrophages were assessed for expression of actin, DOCK180, and M-DOCK/DOCK2 transcripts using RT-PCR with primers described in *Materials and Methods*. *D*, Activity of the GTPase Rac in macrophage lysates was assessed by pull-down assays using Sepharose coupled with GST-PAK protein. SDS-PAGE and Western blotting with a Rac-specific mAb were used to determine whether Rac was present in pull-downs (Active Rac) or in whole cell lysates (Total Rac) to test whether Rac was present at similar levels in untreated and DX-treated cells.

references therein), present in macrophages, may allow the recruitment of Rac/Crk/DOCK180 specifically to membranes in a manner that facilitates phagocytosis of apoptotic cells and possibly other particles (43). Indeed, in preliminary experiments using low density IgG-opsonized particles, we have found that DX-treated monocytes/macrophages are more efficient phagocytes (untreated, 24.5% phagocytosis; DX treated, 53.5% phagocytosis, $n = 4$). Importantly, these data suggest that expression or phosphorylation of p130Cas may have a negative regulatory role upon macrophage phagocytic potential. We would speculate that decreased expression/phosphorylation of p130Cas together with augmented Rac activity defines a subpopulation of macrophages highly competent for phagocytosis of apoptotic cells.

Interestingly, recent data suggest that dendritic cell capacity for presentation of apoptotic cell-derived material via MHC class I or class II may depend on $\alpha_v\beta_5$-mediated internalization mechanisms (44). Our data suggest that in addition to differences in integrin usage, DX-treated macrophages lack p130Cas, a molecule that is recruited following apoptotic cell binding to dendritic cells (45). One possibility is that p130Cas expression may determine the cellular consequences of apoptotic cell handling in macrophages and other cell types. Recent studies have shown that immature dendritic cells treated with glucocorticoids down-regulate the capacity for production of IL-12, and consequently induce a regulatory phenotype (46–48). Changes in the adhesion status of macrophages described in this work may also have important consequences for other macrophage functions that influence the progression of inflammation. Loss of podosome adhesion structures observed in Wiskott-Aldrich syndrome macrophages leads to defective chemotaxis responses as a result of loss of the capacity for polarization (49). Whether DX-treated macrophages show increased potential for directed migration necessary for recruitment or emigration of cells to and from the inflamed site has not been assessed and may also be dependent on the effects of DX upon expression of chemokine receptors and activation of kinases such as extracellular signal-related kinase 1 and 2. One speculation would be that in addition to augmented capacity for clearance of apoptotic cells, emigration of DX-treated macrophages from inflamed sites to draining lymph nodes might be altered. Formation of podosome structures in osteoclasts has also been shown to require p130Cas/

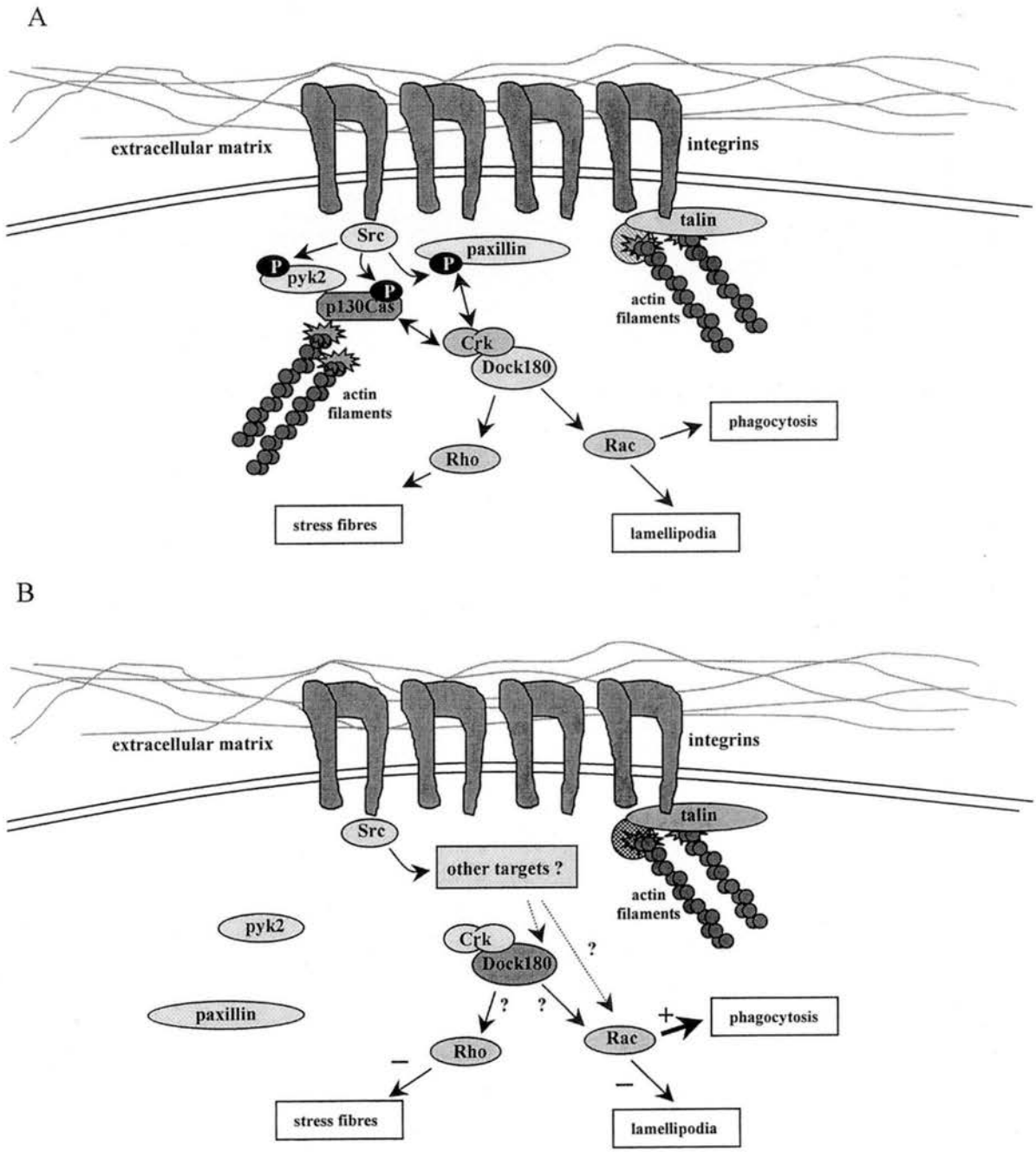

FIGURE 6. Schematic representation of the effects of DX upon cytoskeletal elements present in macrophages. *A*, Attachment to matrix activates a number of adhesion pathways. Integrin ligation induces Src activation and phosphorylation of pyk2 and the scaffolding protein paxillin and p130Cas. The resulting phosphotyrosine motifs recruit Crk and downstream Crk effectors, including DOCK180. The DOCK180/Crk/p130Cas complex may facilitate actin redistribution and podosome formation and may lead to activation of the small GTPases Rac and Rho. *B*, Following DX treatment, loss of phosphorylation of paxillin and pyk2 would be expected to disrupt interactions of these molecules with other effectors. Crk and DOCK180 complexes that are required for formation of adhesion structures may be altered by the reduced levels of p130Cas. Our data would suggest that activation of Rac is facilitated despite the reduced levels of p130Cas in DX-treated cells.

Crk/DOCK180 (50). One interesting possibility is that the detrimental effects of prolonged steroid treatment on bone homeostasis may be mediated though disruption of important adhesion events associated with bone homeostasis.

A striking feature of DX-treated macrophage phenotype was the lack of large multinucleated cells observed in culture when compared with untreated cells. Preliminary data indicate that formation of multinucleated macrophages promoted by treatment with IFN-γ (51), or following stimulation with CD98 (52), is inhibited by DX, consistent with data on alveolar macrophages (53), and suggests a dominant regulatory effect of glucocorticoids upon macrophage differentiation. Although previous reports have suggested that glucocorticoids drive monocyte apoptosis (54), we believe that these apparently discrepant results are accounted for by differences in the culture media used. In this present study, monocytes were cultured in 10% autologous serum with no differences in the numbers of cells recovered with or without DX (control, 152 ± 16 cells/field; DX, 140 ± 16 cells/field; average counts ± SEM from 10 separate experiments). Moreover, the phenotype we observe is unlikely to represent selection of a subpopulation of monocytes that

are resistant to glucocorticoid-induced death. The percentage of monocyte-derived macrophages that phagocytose apoptotic cells is increased at least 3-fold by DX treatment, requiring that most of the cells in the initial population be lost if selection of a phagocytic phenotype occurred. Although production of modulatory cytokines (e.g., TGFβ) following DX treatment might influence monocyte differentiation and phagocytic potential, exposure of monocytes to TGFβ or IL-10 does not induce the prophagocytic functional phenotype described in this study (not shown).

In conclusion, we report the novel finding that early exposure of monocytes to glucocorticoids induces a proresolution phenotype. The proportion of monocytes/macrophages capable of phagocytosis of apoptotic cells and the phagocytic index are increased dramatically following DX treatment. The combined effect would give rise to a markedly enhanced potential for clearance of apoptotic cells from an inflammatory site following DX treatment, with considerable implications for therapeutic strategies for manipulation of inflammatory processes in vivo. The prophagocytic phenotype of DX-treated monocytes/macrophages was characterized by profound morphological changes, down-regulation of phosphorylation of paxillin and pyk2, and loss of p130Cas expression. We propose that the increased Rac activity we observe together with these changes in cytoskeletal changes may define a prophagocytic macrophage phenotype. These data further emphasize the importance of understanding the contribution of adhesion-related signaling pathways in the regulation of macrophage phagocytosis.

Acknowledgments

We thank our colleagues in the Centre for Inflammation Research for their help with cell isolation and other aspects of this work; in particular, Dr. Graham Thomas for help with microscopy, Dr. Sandrine Prost for video microscopy, and Stephen Mitchell for electron microscopy.

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